MARKER ASSISTED SELECTION FOR HEAT TOLERANCE IN RICE (*Oryza sativa* L.)

By,

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(2017-11-021)

THESIS

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DECLARATION

I, hereby declare that the thesis entitled '**Marker assisted selection for heat tolerance in rice (*Oryza sativa* L.)**' 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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CERTIFICATE

Certified that the thesis entitled **'Marker assisted selection for heat tolerance in rice (*Oryza sativa* L.)'** is a record of research work done independently by **Ms. Silpa V.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

%	Per cent
>	More than
BSA	Bulk Segregant Analysis
BLB	Bacterial leaf blight
BC_1	1st backcross generation
BC_2	2nd backcross generation
bp	basepairs
cm	Centimetre
cM	Centimorgan
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy Ribo Nucleic acid
EMF	Early Morning Flowering
F_1	1st filial generation
F_2	2nd filial generation
F_3	3rd filial generation
F_4	4th filial generation
F_5	5th filial generation
g	Gram
GGT	Graphical Geno Types
Kg	Kilogram
IRRI	International Rice Research Institute
MABB	Marker assisted backcross breeding
MAS	Marker assisted selection
Max.	Maximum
Min.	Minimum
MSL	Mean Sea Level
µg	Microgram
µl	Microliter
ml	Milliliter
mm	Millimeter

mM	Millimolar
No.	Number
N22	Nagina22
OD	Optical Density
PCR	Polymerase chain reaction
RM	Rice microsatelite
SSR	Simple sequence repeats
TAE	Tris Acetic acid EDTA

Introduction

I. INTRODUCTION

Rice is the staple food crop of Kerala. Rice production in the state is decreasing owing to the declining trend in rice cultivation. Various biotic and abiotic stresses triggered by climate change are but a few reasons behind the steep decline. Temperature increase globally is estimated to be 1.1 °C to 6.4 °C during the next century (IPCC, 2014). High temperature stress is a major constraint in rice production in tropical and subtropical regions. High temperature results in heat induced spikelet sterility in rice. Rice yields are estimated to be reduced by 41 per cent due to high temperature at the end of the 21st century (Ceccarelli *et al.*, 2010).

High temperature stress in plants is a complex function of heat intensity, rate of increase in temperature, and duration of heat exposure (Wahid *et al.*, 2007). Current adaptations to high temperature *via* alterations to technical and management systems are insufficient to sustain the yield. In this context, genetic improvement of heat stress tolerance traits of staple crops especially rice is of immediate necessity.

In the major rice growing tracts of Kerala *viz.*, Palakkad, Kole, and Kuttanad, the temperature tends to rise up to 39 °C or even more, especially during the second/third cropping season. Consequently, high temperature induced sterility has become a serious problem.

To tackle this, high yielding varieties coupled with heat tolerance suited for our fields must be developed urgently. Since most of the prevailing high yielding rice varieties like Uma are highly susceptible to heat stress, it is essential to impart heat tolerance to such varieties which are cultivated to very large extent in Kerala. With advancements in molecular marker technology, imparting heat tolerance to high yielding varieties from potential donors can be hastened through marker assisted selection.

Marker assisted selection (MAS) has been identified as a dependable, reproducible, and time saving strategy to confirm the presence of desirable gene and to quicken the breeding cycle. Marker assisted selection is having considerable importance in breeding genotypes for adaptation, as it involves high precision in gene/QTL transfer (foreground selection), accelerates recurrent parent genome recovery (background selection), and restricts the DNA fragment to desired size (recombinant selection). Due to the difficulty of managing heat tolerance traits through conventional phenotypic selection and the presence of several QTL for such a polygenic trait with complex inheritance, MAS is considered as an efficient method for screening rice genotypes for heat stress.

The aus *indica* type rice variety 'Nagina22' (N22) has been characterised for several heat stress tolerant traits (Bahuguna *et al.,* 2015). The variety is early maturing with high capacity for regeneration and recovery processes, and flexibility in the accumulation and mobilization of carbohydrates (Gorantla *et al.,* 2006). However, relatively small-sized grains and weak and long stems, a trait that leads to lodging of the plant and loss of grain yield are a few undesirable traits of N22 (Bahuguna *et al.*, 2015). The tolerance to heat stress is a prominent trait associated with this variety that can be leveraged to identify genetic regulation of heat stress tolerance in rice. The heat-stress tolerant traits from N22 can be used for introgression into other varieties for developing climate-change ready rice (Ye *et al.,* 2012).

In this context, study was conducted for the identification of SSR markers linked to the genes for heat tolerance in rice through bulked segregant analysis approach using F_3 population of the cross Uma x N22 at College of Horticulture, Vellanikkara. The RM marker, RM5749 was found to be tightly linked to spikelet fertility trait under heat stress (Gorakh, 2017).

In continuation of this work, the present research programme was carried out with the objective of selection of plants for heat tolerance in F_4, F_5, and the backcross

populations generated using the selected tolerant lines of the cross Uma x N22 with high yielding parent (Uma). Genotyping of selected F_4 and F_5 lines of the cross Uma x N22 using RM5749 and background markers for Uma were also included in the present study.

Review of literature

II. REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is the most widely consumed food crop in the world. Rice yield is subjected to severe losses due to adverse effect of a number of biotic and abiotic stress factors. Climate change has increased the intensity of heat stress which results in serious economic losses of agricultural and horticultural crops. (Beck, 2013) Productivity of rice, world's most important cereal is threatened by high temperature stress, which is intensified by climate change. Flowering and seed development stages of rice crop is sensitive to high temperature stress and produce sterile spikelets, unfilled and chalky grains which decrease the quantity and quality of rice seeds. Development of heat stress-tolerant varieties is one of the best strategies to maintain its productivity. However, heat stress tolerance is a multigenic trait and the candidate genes are poorly known. The use of yield as a selection criterion for the development of stress tolerant varieties is usually prohibitive in early generations. Hence, methods such as marker assisted selection is a better alternative.

2.1 Climate change and crop yield in rice

Global warming has become one of the most complicated problems affecting agricultural productivity. Agriculture will be affected by climate change owing to higher temperatures (estimated to increase +2 °C by 2050), changing rainfall patterns and higher carbon dioxide (CO_2) levels. Jaggard *et al.* (2010) predicted that a change in weeds, pests and disease pressure on crops will also be associated with these climatic changes.

Global emissions of carbon dioxide caused by human activities reached a record high in 2011 and is likely to increase in succeeding years, thus contributing to the global increase in temperature (Smith and Olesen, 2010). Global climate model projections summarized in the 2007 Fourth Assessment Report (AR4) by the Intergovernmental Panel on Climate Change (IPCC) indicated that during the 21st century, global surface temperature is likely to rise by 1.1 to 2.9 °C for their lowest

CO_2 emissions scenarios and by 2.4 to 6.4 °C for their highest emissions scenarios. Baker *et al.* (1992) estimated 7-8 per cent rice yield reduction for each 1°C increase in day temperature from 28 °C to 34 °C.

Summer heat stress has become an important environmental factor in limiting rice yield with the intensified climate change (Espe *et al.*, 2017). Rice yields have been estimated to be reduced by 41 % due to temperature stress by the end of 21st century. (Ceccarelli, *et al.*, 2010). Lobell *et al.* (2017) predicted that rice yield will decrease by 20-40 per cent in the tropics and 3.1 per cent (19 million metric tonnes) in subtropics by the end of this century.

Ishimaru *et al.* (2015) observed that in Laos and southern India, the combined stresses of heat and intense solar radiation during daytime aggravate the spikelet sterility of local popular cultivars when heading coincides with high temperatures.

2.2 Effect of high temperature on various growth stages of rice

Although rice can still maintain normal growth at temperatures ranging from 27 to 32 °C without significant reduction in grain yield, temperatures above 32 °C negatively affect all stages of rice plant growth and development (Aghamolki *et al.*, 2014) and the most critical temperature was found to be 33 °C during the flowering stage (Jagadish *et al.*, 2007).

High temperature affects most physiological processes, including stomatal opening, photosynthesis, growth and grain yield. Heat tolerance studies in rice focused mainly on the reproductive stage, as they were extremely sensitive and immediately relevant for grain yield (Prasad *et al.*, 2008; Jagadish *et al.*, 2010; Aghamolki *et al.*, 2014; Das *et al.*, 2014; Hatfield and Prueger, 2015).

Shah *et al.* (2011) reported that flowering and booting stages of rice are most susceptible to high temperatures. Guilioni *et al.* (1997) observed that even a short

period of heat stress can cause significant increase in the abortion of floral buds and opened flowers during the reproductive stage.

Dwivedi *et al.* (2015) studied the effect of rising CO_2 concentrations and elevated temperature on yield and yield contributing characters in rice. Physiological characters like membrane stability index per cent, chlorophyll content, photosynthetic efficiency, and relative water content were not affected and all rice cultivars performed better for these traits under elevated CO_2. But these traits were observed to be negatively affected at elevated temperature. They concluded that elevated CO_2 had a positive effect while, high temperature negatively affected the grain yield of rice by altering normal metabolism.

2.3 Effect of high temperature at vegetative stage

According to Osada *et al.* (1973), plant height increases within a temperature range of 30-35 °C. Han *et al.* (2009) reported that when rice seedlings were exposed to high temperatures (35, 40 and 45 °C) for 48 hours, the maximal quantum yield of photosystem II (PS II), photochemistry, the activity of ascorbate peroxidase, and the proteome changes were more at higher temperature.

Forty rice genotypes including 23 introgression lines (IL) derived from BC_2F_6 of Swarna × *O. nivara* and KMR3 × *O. rufipogon* and three mutants of N22 were evaluated for heat tolerance at germination, seedling and early vegetative stage of development under different temperature regimes (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 per cent germination, mean shoot and root length, dry weight, chlorophyll, carotenoids and leaf senescence index compared to the tolerant check Nagina 22 at high temperature. At 40 °C, two deep water rice varieties, Jalmagna and Madhukar and IL117 showed highest values for germination, mean shoot and root length, dry weight. The two N22 mutants

NH686 and NH787 had the least shoot and root length at 40 °C. The effect of heat on carotenoid content was highly significant.

Pale *et al.* (2012) evaluated a total of 600 rice germplasm from the North-Eastern hills of India for their tolerance to high temperature. A total of 78 genotypes showed more than 80 % germination at 40 °C, while only 27 genotypes showed 60 % or more germination at 45 °C. After heat treatment of the latter (27 genotypes) at 40 °C and 45 °C at the seedling stage, eighteen genotypes successfully recovered from the 40 °C treatment, while only nine recovered after exposure at 45 °C. These nine genotypes were found to contain 30-60 % relative water content under 22 days of drought stress.

When rice is exposed to higher air temperatures during the vegetative stage, individual plant height, tiller number and dry weight may be considerably reduced (Bhattacharya and Ali, 2016).

2.4 Effect of high temperature at reproductive stage

Flowering is the sensitive growth stage of rice to heat stress (Mackill *et al.,* 1982; Kuang *et al.*, 2002). A high temperature of beyond 35 °C during flowering can induce floret sterility and consequently high yield losses (Osada *et al.*, 1973; Stake and Yoshida 1978; Matsushima *et al.*, 1982), and it may lead to 80 % loss in yield (Li, 2003). Rieu *et al.* (2017) pointed out that formation of the male gametophyte (pollen) is more sensitive to the growing environment. A temperature of 33.7 °C for an hour at anthesis induces spikelet sterility (Jagadish *et al.,* 2007). Due to high temperature during flowering, rice produces chalkiness due to poor starch packing that breaks the rice grains during milling (Collis and Braidotti, 2009).

Sexual organogenesis in spikelets of rice affected by heat stress was investigated by Takeoka *et al.* (1991) using SEM and stereo-microscopy in 243 spikelets of *Oryza sativa* L. cv 'Kinmaze'. The structural development of the anther was observed to be disturbed, and microsporogenesis was found to be inhibited. The

study suggested that elevated temperatures cause pistil hyperplasia in rice, which leads to spikelet sterility and stamen hypoplasia.

Xian-Yong *et al.* (2008) studied the effect of high temperature on seed setting in rice. The seed setting rate was negatively related to temperature when high temperature occurred on the day before, during or one day after flowering. The seed setting per cent was highest when high temperature was one day after flowering. Seed setting was lesser in plants when high temperature occurred on flowering day than in plants which exposed to high temperature one day before flowering. To get normal seed setting rate (> 80 %), high temperature tolerant combinations need less than 34.8 °C, high temperature sensitive combinations need less than 34.4 °C. The difference of tolerance to high temperature among different hybrid combinations was highly significant when the temperature was between 37 and 40 °C.

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

Liu *et al.* (2013) studied the effects of high air temperature from R3 (Panicle exertion from boot) to R8 stage (physiological maturity) on rice grain yield and quality in two rice cultivars, Japonica type Koshihikari and Indica type IR72. They found that percentage of chalky kernel increased whereas, yield, amylase and starch content and grain yield decreased in both cultivars under high air temperature.

Nguyen *et al.* (2013) constructed a simulation model for predicting spikelet sterility with response to high air temperature. The model was based on estimates of heading date of panicles in the field which followed Poisson distribution, flowering date of spikelets on a panicle which followed normal distribution, flowering time of spikelets during the day time and the two sterility response functions to the temperature on the day of meiosis and at the flowering time of a spikelet. Spikelet fertility reduced with temperature rise at meiosis and flowering. The heating degree hour causing 50 % spikelet sterility at meiosis was higher in a *japonica* cultivar 'Hwaseongbyeo' than in a *Tongil* type 'Dasanbyeo', while the air temperature causing 50 % spikelet sterility at spikelet flowering time was higher in *Tongil* type cultivars than in *japonica* ones.

Das *et al.* (2014) studied pollens of two groups of genotypes subjecting the plants to six different day/night temperature regimes with a constant diurnal temperature variation of 10 °C for three days during panicle initiation. High temperatures above 35/25 °C adversely affected panicle extrusion, flowering period, and number of opened spikelets. Significant decrease in viability and tube length of pollens, poor anther dehiscence and reduced number of pollens on stigma were observed. Notably, the adverse effect of high temperature stress was more on the pollens of lowland genotypes than on those of upland genotypes. The study suggested that tolerance to higher temperature stress during the development of reproductive organs varied, with viability of pollens distinctly better in the upland genotypes than the lowland genotypes.

2.5 Heat tolerance in rice

To date, screening for heat tolerance has been conducted in the environmentally controlled chambers and open fields in heat-vulnerable regions. In the chamber experiments, heat tolerance at flowering is often tested at 37.5–38.0 °C with relative humidity around 60–70 % to have a great contrast in spikelet fertility between

susceptible and tolerant genotypes (Kobayasi *et al.*, 2011; Mackill *et al.*, 1982; Shi, 2015). Like many other crop species, there is substantial genetic variations in rice germplasm exists which can thrive better under the prevailing high temperature environments (Shah *et al.,* 2011). N22 and Dular are considered as highly tolerant genotypes to heat stress at flowering stage (Jagadish *et al.*, 2010; Buu *et al.,* 2014).

Guilian *et al.* (2007) studied the effects of heat stress on the flower morphology, yield components and grain quality of two rice cultivars. Heat stress significantly affected the glume opening angle, filament length, pollen diameter, chalkiness and protein content and reduced anther dehiscence, percentage of stainable pollen grains, seed set rate, 1000 grain weight, milky rice rate, head rice rate, alkali spreading value, gel consistency and amylase content, but did not have significant effects on stigma vigour and number of grains per panicle. The grain quality of the heat sensitive cultivar was highly affected by heat stress.

Bo *et al.* (2012) studied heat resistance during flowering in 100 hybrid combinations. The rice plants were subjected to heat stress at 38 °C for 3 days as 50 % spikelets flowered. Based on relative spikelet fertility rate, they were classified into heat tolerant hybrids, semi-heat tolerant hybrids and heat sensitive hybrids. Y Liangyou 646, Guangzhan 63-45 x R558, Xixang 5979, Y Liangyou 2 and Y Liangyou 896 were heat tolerant hybrids, whereas 11 you 838, Fengyou 199 and Lingchugou 37 were found to be heat sensitive hybrids. Analysis of origin of combinations with different heat tolerance indicated that most hybrids derived from the same female parent showed similar heat resistance. It indicated female parent played a more important role in heat tolerance of hybrid rice.

Rani and Maragatham (2013) conducted an experiment to study the influence of elevated temperature on rice phenology and accumulated growing degree days during Kharif 2012 under temperature control chamber in which temperature is elevated from the ambient level (2 °C and 4 °C) for the entire crop growth period. The results showed

that the days taken to attain maturity was less under elevated temperature of 4 °C (96 days) and 2 °C (102 days) when compared to the ambient temperature (108 days). The accumulated growing degree days were higher under elevated temperature of 4 °C and nearer value for 2 °C viz., 1641 and 1583 respectively from that of ambient. Under elevated temperature of 4 °C and 2 °C, the grain yield was 23 and 13.3 per cent respectively less than the ambient condition. The highest grain yield was from the treatment under ambient temperature with 6.2 t/ha followed by 5.3t/ha under 2 °C level and 4.7t/ha at 4 °C level. The yield loss under elevated temperature was due to the sterile florets and lesser crop duration.

Tenorio *et al.* (2013) selected 200 accessions with high spikelet fertility for phenotyping in temperature controlled outdoor growth chambers with 38/21 °C and 70/75 % day/night relative humidity. They identified 23 accessions as potential donors for heat tolerance. The donors were further subjected to high temperature treatment of 39 °C at booting stage and 38 °C at flowering stage. Based on spikelet fertility percentage and grain yield per plant, Dular and Todorokiwase were found to be tolerant at the booting stage, while Milyang23 and IR2006-P12-12-2-2 were tolerant at the flowering stage. Only Giza178 was found to be tolerant at both booting and flowering stages.

Cheabu *et al.* (2018) tested seed-setting rate, yield components and grain quality traits of 169 accessions from an exotic rice germplasm under high temperatures from 40 ºC to 45 ºC for 6 h during the daytime at the vegetative (45 d before heading) and reproductive stages (15 d before heading), respectively. The results showed that the seed-setting rate of all the accessions significantly decreased under heat stress. Based on the decrease in seed-setting rate at high temperatures, N22 was adjudged to be the most tolerant, followed by Aus17, M9962, Sonalee and Aus16. The reductions in seed-setting rate and yield under heat stress were more serious at the vegetative stage than at the booting stage. Heat stress also affected grain quality, especially by conferring chalkiness to most of the accessions, but the change was less in Sonalee.

2.6 Mechanisms of heat tolerance in rice

Since the plants are immobile in nature and exposed to persistently changing environmental conditions, they have evolved multiple mechanisms to cope up stressful unfavorable environmental conditions to optimize its growth within the defined temperature ranges (Crawfod *et al.,* 2012). Plant survive heat stress through either heat tolerance or avoidance mechanism.

Anther dehiscence requires rupture of cell layers, septum and stomium which keeps the adjacent locules closed. Opening of the locules apparently results in pollen release from the anthers. High temperatures at flowering inhibit swelling of the pollen grains, which is the driving force behind anther dehiscence in rice (Matsui *et al.*, 2002). Anthers of high temperature-tolerant cultivars dehisce more easily than those of susceptible cultivars and contribute to pollination under high-temperature conditions (Mackill *et al.*, 1982; Matsui *et al.*, 2000, 2001).

Ishimaru *et al.* (2010) found that high temperatures over 32-36 °C at anthesis induce spikelet sterility in rice. In this study, the effect of the EMF (Early morning flowering) trait on avoiding high temperature induced sterility at anthesis by flowering at a cooler temperature in the early morning was evaluated. The EMF trait was introgressed from wild rice (*Oryza officinalis*) into the rice cultivar Koshihikari (*O. sativa*). In a greenhouse experiment, spikelets of Koshihikari opened after the air temperature reached 35 °C, but those of the EMF line could open at cooler temperatures. Under these conditions, spikelet sterility significantly increased in Koshihikari, but did not in the EMF line. The early morning flowering trait of wild rice is effective in mitigating anticipated yield loss due to global warming by escaping high temperature stress at anthesis during the day time.

Recently, Bheemanahalli *et al.* (2017) demonstrated opening of the spikelets early in the morning as an useful criterion for selecting heat tolerant rice plants/genotypes. In their study, 289 diverse rice cultivars from tropics and subtropics

rice growing regions were evaluated over three years in wet and dry seasons. EMF traits such as first spikelet opening time and peak spikelet opening time were quantified among these cultivars. A NIL (IR64 +qEMF3) reduced spikelet fertility by 71 per cent during dry seasons in field compared to the 289 rice cultivars used for the study indicating the usefulness of incorporating EMF trait in reducing heat stress induced sterility under field conditions. They concluded that genotypes which flowers open early in the morning is utilizing the avoidance mechanism have better heat tolerance.

The heat-tolerant accessions exhibiting tolerance at booting and flowering stage are considered to be useful genotypes for the breeding programme to improve heat resilience in terms of spikelet sterility. Flowering at cooler times of the day, higher pollen viability, bigger anthers, extended basal dehiscence and presence of extended basal pores are considered to be morphological markers for high temperature stress tolerance. Reduced evaporation rate results in swelling of pollens which is a crucial mechanism of anther dehiscence. Hence, the cultivars with covered panicles are better tolerant to high temperature because of their ability to reduce the evaporation rate from anthers - reduced the spikelet sterility (Shah *et al.*, 2011).

Hasanuzzaman *et al.* (2013) reported that heat stress triggers the expression of certain genes and metabolites production enhancing the heat tolerance in plant. In rice, the tolerance to heat is linked with the production of high RNA content, strong antioxidative defense system, and less malondialdehyde content (Anjum *et al.*, 2016) during meiosis (Cao *et al.*, 2008). Protection of structural proteins, enzymes, and membranes and expression of heat shock proteins are some of the biochemical processes that can impart thermotolerance (Shah *et al.*, 2011).

2.7 Varietal selection

Nagina22 (N22) has been characterized for several heat stress tolerant traits (Jagadish *et al.*, 2007; Ye *et al.*, 2012; Bahuguna *et al.*, 2015). Bahuguna *et al.* (2015) identified that this variety has some undesirable traits like relatively small-sized grains and weak and long stems, a trait that leads to lodging of the plant and loss of grain yield. N22 possesses desirable morphological and physiological characters like early maturity, high capacity for regeneration.

Rang *et al.* (2011) reported that tolerance to heat and a combination of heat and water deficit stress is reported only in Nagina22. Similar response has not been documented for tolerance to either of the stresses by any other entries.

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. Hence, ability to shed pollen at high temperature is ineffective in the selection of cultivars for high temperature tolerance. (Jagadish *et al.*, 2010)

Manigbas *et al.* (2014) reported that heat tolerant varieties such as N22 and Dular, usually have low yield potential and undesirable plant characteristics but combining them with high yielding and improved rice varieties, new heat tolerant rice genotypes with high yield potential can be developed. In this study, phenotyping and selecting desirable materials from various crosses were carried out under high temperature conditions during the reproductive stage in glass house and field. Several advanced breeding lines from Gayabyeo/N22 cross produced desirable individuals with heat tolerance, resistance to pests and diseases and high yield potential. The genetic variation in per cent sterility among the selected backcross populations grown in high temperature environments showed that large number of plants can be identified and selected with lower per cent sterility.

2.8 Marker assisted selection

Rapid advances in genome research and molecular technology have led to the use of DNA marker assisted selection which enhances the selection efficiency for the development of cultivars with higher yield potential (Ribaut and Hoisington, 1998). While marker assisted foreground selection helps in identifying the gene of interest without extensive phenotypic assays, background selection can significantly increase the rate of genetic gain or recovery of recurrent parent genome in a backcross breeding programme. (Tanksley *et al.,* 1983). Marker assisted foreground selection was proposed by Tanksley (1983). Melchinger (1990) investigated foreground selection in the context of introgression of resistance genes and presented a priori approach for the minimum number of individuals and family size required in recurrent backcrossing. Hospital and Chacrosset (1997) coined marker assisted background selection. They investigated the combined foreground and background selection for introgression of favourable genes at quantitative trait loci.

Reliability, quantity and quality of the DNA required, technical procedure for marker assay, level of polymorphism and the cost are the main factors to be considered for the use of DNA markers in MAS. (V. Mohler *et al.,* 2004). Moreover, the success of MAS depends on several other factors, including the number of target genes to be transferred and the distance between the flanking markers and the target gene (Perumalsamy *et al.*, 2010).

Gupta and Varshney (2000) surveyed that Simple sequence repeats (SSRs) or microsatellites are the widely used markers in major cereals. Gao *et al.* (2016) also reported that currently SSRs (second-generation markers) are widely used markers in MAS due to the easy availability and comparatively cheaper than others and they require a comparatively simple technique with a higher polymorphism rate.

A major challenge in successfully deploying a molecular breeding system is the non-availability of robust markers for every target trait, especially for QTL (Cobb *et*

al., 2019). Another challenge is the limited population size in the segregating generations due to poor seed set especially observed in leguminous crops, more specifically in MABB, which may impede the rapid recovery of genome as well as phenome. (Singh *et al.,* 2019)

2.9 Marker assisted selection for heat tolerance in rice

In a study conducted by Xiao *et al.* (2011) two QTL underlying tolerance to high temperature stress were identified using recombinant inbred lines derived from a cross between a heat tolerant rice cultivar 996 and a sensitive cultivar 4628. Pollen fertility was used as a heat-tolerance indicator for the lines subjected to high temperature stress at the flowering stage in field experiments. Two QTL that affected pollen fertility, qPF4 and qPF6, were detected between RM5687 and RM471 on chromosome 4, and between RM190 and RM225 on chromosome 6, by using the composite interval mapping (CIM) analysis.

Ye *et al.* (2012) observed high spikelet fertility in control and high temperature treated plants of IR64 (94 %) and N22 (95%). In the treatment of high temperature stress, spikelet fertility of IR64 (21%) was very lower than that of N22 (81%), whereas it ranged from 0 per cent to 89 per cent in 158 F_2 progenies. A study was conducted to map QTL for heat tolerance at flowering stage in rice using SNP markers by Ye *et al.* (2012). BC_1F_1 and F_2 populations derived from an IR64 x N22 cross were exposed to 38/24°C for 14 days at the flowering stage, and spikelet fertility was assessed. Four putative QTL were found to be associated with heat tolerance in the F_2 population. Two major QTL were located on chromosome 1 (qHTSF1.1) and chromosome 4 (qHTSF4.1). The effect of qHTSF4.1 on chromosome 4 was confirmed in selected BC_2F_2 progenies from the same IR64 x N22 cross, and the plants with qHTSF4.1 showed significantly higher spikelet fertility than other genotypes.

Poli *et al.* (2013) characterized EMS induced N22 leaf mutant NH219 and mapped yield traits in 70 F_2 segregants of IR64 X NH219 and 36 F_2 segregants of its

reciprocal cross. NH219 is more heat tolerant than wild N22 as its per cent yield reduction was lesser than N22. Single marker analysis showed significant association of RM1089 with number of tillers and yield per plant, RM423 with leaf senescence, RM584 with leaf width and RM229 with yield per plant.

Bharathkumar *et al.* (2014) screened seven rice landraces *viz.* Rupsal, Nagalmutha, Ravana, Marishal, Polai Talmugra and Raspanjar for heat tolerance at flowering stage with three SSR markers RM3735, RM3586 and RM6100. Expression of OsDREB2A gene which is associated with induction of heat shock-responsive genes was found to be induced strongly in Marishal genotype and this genotype was documented for possessing unique gene allele with RM6100 marker. RM6100 marker is linked with a major QTL on chromosome 10 for heat stress tolerance at flowering stage (Xiao et al. 2011).

Buu *et al.* (2014) evaluated 310 BC_2F_2 lines derived from the cross of OM5930/N22 for heat stress at flowering. Genetic map was set up with 264 polymorphic SSRs to detect linkage to the target traits. The map covers 2,741.63 cM with an average interval of 10.55 cM between two marker loci. Markers associated with heat tolerance were located mostly on chromosomes 3, 4, 6, 8, 10 and 11. Four QTL were detected for filled grains per panicle on chromosome 4 at the interval of RM468 - RM7076 and RM241 - RM26212. Two QTL controlling unfilled grain was also detected at loci RM554 and RM3686 on chromosome 3. One QTL was detected for 1,000-grain weight located at the locus RM103 on chromosome 6. Also, a QTL at the locus RM5749 on chromosome 4 was identified which explained 10.8% of the total phenotypic variance of grain yield. A single QTL at the interval of RM3586-RM160 on chromosome 3 was detected in conformity with the QTL findings for heat tolerance as in previous studies.

Lang *et al.* (2015) used five *indica* varieties, OM5939, AS996, IR66, Gayabyeo (high yielding and susceptible to heat) and IKO547 (resistance to brown planthopper

possessing *Bph18*) were used as recurrent parents to develop heat tolerance lines. N22 and Dular were used as donor of QTL for heat tolerance. They used two markers RM3586 and RM160 on chromosome 3 and four markers RM3735, RM3471, RM3687 and RM3536 on chromosome 4 to select promising lines in backcrossing populations for heat tolerance at flowering stage in rice. To evaluate heat tolerance at the reproductive period fifty lines selected in BC_3F_2, BC_4F_1, and BC_4F_2 and parents, the genotypes were planted in field under natural heat stress and greenhouse. Heat tolerance scoring under field condition was based on percentage of unfilled grains. All selected lines exhibited their homozygous alleles with two heat tolerance germplasm N22 or Dular in QTL loci. Twelve lines harboring homozygous alleles to QTL loci RM3586 on chromosome 3 and RM3735 on chromosome 4, respectively were selected and evaluated to agronomic traits and yield potential. Based on MAS, phenotypical acceptability, yield components and yield, four backcross lines were finally selected.

Shanmugavadivel *et al.* (2017) phenotyped a RIL population of 272 F_8 recombinant inbred lines created between N22 and IR64 for spikelet sterility and yield under heat stress and mapped multiple QTL using a linkage map with 824 SNP markers. They identified five QTL on chromosomes 3, 5, 9 and 12. Of these five QTL, two high effect QTL, were mapped in narrow physical intervals (less than 400 Kbp genomic regions) comprising of 65 and 54 genes, respectively. It is the first report of a major QTL for heat tolerance on chromosome 9 of rice. Further, a known QTL for heat tolerance on chromosome 5 was narrowed down from 23 Mb to 331 Kbp in this study. Two major QTL identified in the study for heat tolerance in rice can be employed for crop improvement by marker assisted selection (MAS) after development of suitable scorable markers for breeding of high yielding heat tolerant rice varieties.

Kilasi *et al.* (2018) genotyped 150 F_8 recombinant inbred lines (RILs) of cross N22 x IR64 to identify QTLs and candidate genes for seedling growth under heat

stress. They used genotyping-by-sequencing approach to generate single nucleotide polymorphic (SNP) markers and a linkage map was constructed using 4,074 high quality SNP markers. The study identified ten QTL for heat stress tolerance on chromosomes 1, 2, 3,4,5,6, and 10 similar to studies that identified multiple QTL for heat tolerance during flowering scored by spikelet sterility. Genes coding 1,037 potential transcripts were identified based on their location in 10 QTL regions for vegetative stage heat stress tolerance.

2.10 Marker assisted backcrossing

Marker assisted backcrossing (MABC) is one of the most promising approaches is the use of molecular markers to identify and select genes controlling resistance to those factors. In this regard, MABC can contribute to develop resistant or high-yielding or quality rice varieties by incorporating a gene of interest into an elite variety which is already well adapted by the farmers. (Hasan *et al.*, 2015). The selection of molecular markers intended for MABC can be based on (i) their genome distribution; (ii) haplotype diversity and/or polymorphic information content indices; and (iii) their association with candidate genes and other agronomic traits (excluding target introgression trait) (Xu, 2003; Varshney *et al.*, 2005).

Tanskley *et al.* (1989) worked out that by MABC 90 per cent of the recurrent parent genotype can be recovered within 2 generations when suitable number of markers i.e., one marker at every 10 cM and adequate number of progenies were used for background selection. Babu *et al.* (2004) considered the number of target genes, the distance between the flanking markers and the target gene (2-20 cM) and the number of genotypes selected in each backcross generation as critical factors in a MABC programme. They suggested that once the threshold of one marker per every 20 cM is reached, additional markers except around the target locus is not required. Increasing the number of markers genotyped at each generation had little effect.

Jain *et al.* (2014) reported that marker assisted technologies like MABC and MARS (Marker Assisted Recurrent Selection) are the powerful tools for plant breeders for identification and selection of physiological traits like canopy temperature, stay green habit, chlorophyll content, leaf conductance and grain yield to improve cultivars for heat and drought stress tolerance.

Marker assisted backcross breeding had been successfully applied in many crops. Rice was improved through MABC for bacterial leaf blight resistance (Joseph *et al.*, 2004 and Sundaram *et al.,* 2008) and for submergence tolerance (Neeraja *et al.*, 2007). Submergence tolerance QTL sub 1 was transferred through MABB strategy to Swarna background in rice. Foreground, background, recombinant selections were carried out in the study using SSR markers. In BC_3F_2 generation, double recombinant lines were identified for sub 1 QTL linked marker as well as homozygous alleles of recurrent parent for other unlinked markers. The lines positive for sub 1 QTL were selected and they exhibited tolerance to submergence under field conditions for 14 consecutive days. They showed 96.2 percent recovery for Swarna type. Based on the findings of the study they suggested that, MABB strategy is an effective tool to reduce number of backcross cycles to three generations as compared 6-8 generations in conventional backcross breeding.

Marker assisted backcross breeding (MABB) has led to successful development and central release of 14 improved rice cultivars by Central Sub-Committee on Crop Standards, Notification and Release of Varieties (CSCSN & RV) possessing resistance to biotic stresses (BB, blast), tolerance to abiotic stresses (drought and submergence) in the background of popular rice varieties across India. (Singh *et al.,* 2019).

In all, marker assisted technology has created the opportunity to tackle specific component of high temperature stress resistance by identifying, mapping and validating and introgressing allelic sources into elite line for improvement against most threatening global environmental stress. Morpho-physiological characters which are

involved in imparting the stress tolerance mechanism through phenotypic adaptations are also important parameters to be studied. Development and evaluation of genotypes for such traits by conducting appropriate breeding strategies is necessary to secure global food supply and farmers' income.

Materials and methods

III. MATERIALS AND METHODS

The present study was conducted at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during 2017-19. Seven selected F_3 lines of the cross Uma x N22 were used to raise F_4 population. The F_4 population was evaluated for its morphological characters. Twenty six lines from F_4 were selected based on spikelet fertility percentage. Foreground selection was also done in segregating populations F_4 and F_5 of the cross Uma x N22 using RM5749, the identified linked marker for heat tolerance. Background selection in these lines were done using markers reported to be polymorphic between the parents UMA and N22. F_5 population was raised from selected F_4 lines. F_5 population was also evaluated for its morphological characters. Selection was carried out using spikelet fertility percentage in this population and genotyping of these selected lines was carried out using RM5749. Background selection of the selected F_4 genotypes was also carried out.

Attempts were made to develop backcross lines using seven selected lines of F_3 as female parents and Uma as the male parent. This chapter describes details of the materials used and the methods followed in the programme.

EXPERIMENTAL DETAILS

3.1 Experimental location

The experiment was carried out at College of Horticulture, Thrissur, 680656, located 40 m above MSL between 10°31'N latitude and 76°13 E longitude and experiencing humid tropical climate.

3.2 Experimental material

N22 is a deep-rooted drought and heat tolerant *aus indica* type rice variety. It has been reported as a heat tolerant genotype exhibiting high levels of spikelet fertility

at high temperature during anthesis. A hybrid of heat sensitive high yielding variety Uma and heat tolerant variety N22 (Uma x N22) was developed at Regional Agricultural Research Station (RARS), Pattambi during 2014. F_2 population of the hybrid (Uma x N22) constituted six plants. The plant in F_2 with the highest yield was forwarded to F_3 generation. Seven selected lines from the tolerant bulk of F_3 plants of previous study formed the basis of the present study.

3.3 Raising of F_4 and F_5 population

Hundred seeds of seven F_4 lines were sown for germination. Fifty nine survived plants of F_4 generation and seventy five plants of F_5 generation were evaluated. Selection among F_4 population was done based on the spikelet fertility percentage and presence of reported linked marker RM 5749. Twenty-six lines from F_4 were forwarded to F_5.

3.4 Development of backcross populations

Two backcross populations *viz.*, BC_1F_1 and BC_1F_2 were raised by crossing F_4 tolerant lines with Uma as male parent. Staggered sowing of the pollen parent Uma was done at weekly intervals starting from 27th December 2017 to 18th January 2018 and 6th June 2018 to 28th June 2018. The seven lines from F_3 which were reported to be heat tolerant were used as female parents and were backcrossed with the recurrent parent Uma to obtain BC_1F_1 seeds.

3.4.1 Emasculation

Emasculation was done during evening time (after 4 pm). Female parent panicles that exhibited more than fifty per cent emergence of the panicle from the flag leaf were selected for emasculation. The spikelets were further exposed by slightly detaching the leaf sheath from the flag leaf. Very young florets with anther heights less than half of the florets were cut and removed. Mature florets with the height of the

anthers equal and more than half of the florets, which were likely to open on the next day were selected for emasculation. Top one third of each selected floret was clipped off to expose anthers. Then all the six anthers were removed by lifting them out with the tip of forceps prong. The emasculated panicles were covered with butter paper bags, tagged and labelled.

3.4.2 Hybridization

The panicles of the male parent Uma, which were ready to dehisce were selected in the morning by 8.00 am. The full bloomed panicles were gently tapped and the pollen grains were collected into a petridish containing distilled water. Then the collected pollen grains were brushed onto the stigma of emasculated spikelets of female parents. Immediately the pollinated panicles were rebagged. A week after hybridisation, seed set was checked. At maturity, seeds were harvested, dried, and stored in a refrigerator.

3.5 Marker assisted selection

3.5.1 Laboratory chemicals, glassware and equipment

The AR (analytical reagents) grade chemicals (extra pure) and molecular weight marker from Sisco Research Laboratories (SRL) were used in this study. The constituents for PCR reaction mixture like Taq buffer, $MgCl_2$, dNTPs, Taq DNA polymerase, etc. used in this study were procured from Genei Pvt. Ltd., Bangalore. The plastic wares from Tarson India Ltd. were used. The SSR (Simple sequence repeats) primers synthesized by Sigma Aldrich Chemicals Pvt. Ltd., Bangalore were used and RNase were supplied by HIMEDIA.

The present research work was carried out using the molecular biology facilities and equipment available at Department of Plant Breeding & Genetics. For centrifugation, high-speed refrigerated centrifuge (Eppendorf 5804 R) was used. The DNA quality and quantity estimation were done using NanoDrop spectrophotometer (Jenway- Genova Nano). Eppendorf Mastercycler® nexus gradient PCR machine was

used for the DNA amplification. Horizontal gel electrophoresis units by Bio-Rad, USA and Tarson India Ltd. were used for Agarose gel electrophoresis

3.5.2 DNA isolation

Reagents used

1. CTAB extraction buffer (2X)
    a) 2 per cent CTAB (w/v)
    b) 100 mM Tris (pH 8.0)
    c) 20 mM EDTA (pH 8.0)
    d) 1.4 M NaCl
    e) 1 per cent PVP
2. 0.2 % β mercaptaethanol
3. Chloroform: Isoamyl alcohol (24:1 v/v)
4. Chilled Isopropanol (100 %)
5. Ethanol (70 % and 100 %)
6. Sterile autoclaved distilled water
7. RNase (10 mg/ml)

Reagent 1 was autoclaved and separately stored at room temperature.

3.5.3 Procedure for extraction of genomic DNA of rice

Isolation of genomic DNA was carried out by following the CTAB method (Dellaporta *et al.*, 1983). Young and tender leaves (one month after transplanting) were collected from each plant in the early morning. The collected leaves were labelled, immediately covered in aluminium foil and brought to the laboratory in the icebox. The extraction of genomic DNA was done using the following protocol.

1. 0.15 g tender leaves collected were weighed and put in a pre-chilled mortar and pestle.
2. A pinch of PVP, 50 µl of β mercaptaethanol and 1 ml of extraction buffer were added into it and the leaves are ground and made into fine paste.

3. Homogenized samples were transferred to autoclaved 2 ml centrifuge tubes and incubated at 65°C in water bath for 30 minutes with an intermittent gentle inversion of tubes.
4. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min. at 4 °C.
5. After centrifugation, the contents got separated into three distinct layers. The aqueous phase was transferred carefully to autoclaved 2 ml centrifuge tube.
6. To this 2 µl of RNase was added and incubated in the water bath at 37 °C for 20 min.
7. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min. at 4 °C.
8. After centrifugation, the aqueous phase was carefully transferred to a new centrifuge tube. To this, 0.6 volume of chilled isopropanol was added and the tubes were incubated at -20 °C for two hours.
9. After incubation, the tubes were centrifuged at 10,000 rpm at 4 °C for ten minutes.
10. The supernatant was gently poured out, retaining the pellet.
11. The pellet was washed with 70 % ethanol and dried inside the laminar air flow until all the ethanol got evaporated.
12. The pellet was dissolved in 70 µl of autoclaved double distilled water and stored at -20°C.

3.5.4 Quality and quantity estimation of DNA with a spectrophotometer

The quantity and quality of DNA was checked using NanoDrop spectrophotometer (Jenway- Genova Nano). Since the absorption maxima for nucleic acids and proteins are at 260 and 280 nm, respectively, absorbance was recorded at both the wavelengths and purity of the sample was estimated using the OD_{260}/OD_{280} ratio. The DNA sample was considered to be pure if the OD_{260}/OD_{280} value is between

1.8 and 2.0. Values below 1.8 and above 2.0 are due to contamination by protein and RNA, respectively.

Procedure

1. The lid of spectrophotometer has been opened followed by the sampling arm, and the pedestal was wiped with a soft laboratory wipe to remove any dust particles.
2. The reading was set to zero with a blank sample (ADD H_2O which used to dissolve the DNA pellet).
3. Then, 1 µl of the test sample was loaded on to the pedestal and measure option was selected and necessary readings were recorded.
4. The pedestal was wiped clean with 70 per cent ethanol using a soft laboratory wipe after taking the measurements.

3.5.5 DNA dilution for PCR

A concentration of 50 µg/ml was required for carrying out the PCR reactions. DNA samples were diluted so as to obtain the required concentration from 100 µl stock solution using distilled water as per the formula $N_1V_1 = N_2V_2$.

3.5.6 Dilution of the primers

Primer stocks were diluted with ADD H_2O to give 1 M stock solutions. Primer dilutions were done by dissolving primer stock and ADD H_2O in the ratio 1:9.

3.5.7 Preparation of reaction mixture for thermal cycling

The desired number of PCR cycles, time and temperatures for denaturation, annealing (AT) and extension were standardized based on the primers used (Table 1 and 2) and the conditions were programmed and saved in the thermal cycler (model-Mastercycler® nexus gradient PCR, made: Eppendorf). Amplification reaction was carried out in 0.2 ml thin walled flat cap tubes containing the following components.

a. Genomic DNA (50 ng/µl) : 1.0 µl
b. 10X Taq assay buffer B : 2.0 µl
c. MgCl2 : 0.7 µl

d. dNTP mix (2.5 mM of each) : 1.0 µl

e. Taq DNA polymerase (3 units) : 0.3 µl

f. Primer (10 pM) : 1 µl each of forward and reverse primer

g. Chilled autoclaved distilled water : 12.5 µl

Total reaction volume : 20.0 µl

The amplification profile followed was:

a. 94 °C for 4 min. : Initial denaturation

b. 94 °C for 45 sec. : Denaturation

c. 50 °C - 55 °C for 1 min. : Primer annealing

d. 72 °C for 2 min. : Primer extension

e. 72 °C for 8 min. : Final extension

f. 4 °C hold for infinity : Storage

Samples were held at 4 °C in the thermal cycler followed by storage at -20 °C until the contents were loaded on to the gel for electrophoresis.

3.5.8 Gel electrophoresis of PCR products

The PCR amplified products were electrophoresed on 3.6 per cent agarose gel at 80 volts for 45 minutes. A ProxiO 100bp DNA Ladder Plus (SRL) was used and ethidium bromide was used for staining. The gel profile was visualized under UV and was saved for further analysis.

Reagents used

1. Agarose (3.6 %)
2. 50X TAE buffer (pH 8.0)
    - Tris buffer (1 M)
    - Glacial Acetic acid
    - 0.5 M EDTA
3. Tracking/loading dye (6X)
4. Ethidium bromide (stock 10 mg/ml: working concentration 0.5 µg/ml)

Procedure

1. The gel casting tray was placed appropriately in a gel caster and the movable wall was adjusted such that the gel casting tray was closed at both ends. A comb was selected depending on the number of samples to be electrophoresed and positioned on the grooves of the gel-casting tray.
2. The gel was prepared by adding 3.6 g of agarose in 100 ml of 1X TAE buffer in a glass beaker. The mixture was heated in a microwave oven until all the agarose particles were completely dissolved and a clear solution was obtained.
3. Then the solution was allowed to cool down to 40 to 50 °C and an appropriate amount of ethidium bromide was added and mixed well. The warm gel was then poured into the gel-casting tray and left to solidify for 20 min. at room temperature.
4. After 30-40 minutes the combs were removed, gel along with the tray was kept inside the electrophoresis tank unit filled 1 x TAE buffer until the wells are submerged in buffer.
5. The PCR products to be electrophoresed were then loaded into the wells after mixing each sample with 1 µl of 6X gel loading dye. Also, first lane is loaded with suitable molecular weight marker, and second lane with blank.
6. The gel was electrophoresed at 80 volts for 45 minutes until the dye has migrated to two third the length of the gel.

3.5.9 Gel documentation

Documentation of the electrophoresed gel was done under UVITECH fire reader software (Merck, UK) for proper visualization of bands in the electrophoresed gel.

3.5.10 Scoring of primers for all genotypes

The gel profiles of individual SSR primer were carefully observed and scored and this data was used for further analysis.

3.5.11. Molecular weight analysis

The analysis of molecular weight of PCR images was done by using Navigating 1D MAX software, UVITECH Cambridge. In comparison to the known molecular weight marker, the location of amplicon position and its molecular weight were assessed.

3.5.12 Foreground selection

Foreground selection carried out using RM5749, the identified linked marker for heat tolerance in segregating populations F_4 and F_5 of the cross Uma x N22.

Table 1. Marker used in foreground selection (Gramene marker database)

Primer	Sequence		Annealing temperature (°C)	Product size (bp)
	Forward (5'-3')	Reverse (5'-3')		
RM 5749	GTGACCACATCTATATCG CTCG	ATGGCAAGGTTGGATC AGTC	58	162

3.5.13 Background selection

Genotyping of selected selfed lines in F_4 and F_5 generations was done using background markers of Uma. A set of 35 RM markers reported to be polymorphic between the parents UMA and N22 (Gorakh, 2017) were used for background selection.

Table 2. List of primers used for background selection (Gramene marker database)

Primer	Sequence		Annealing temperature (°C)	Product size (bp)
	Forward (5'-3')	Reverse (5'-3')		
RM302	TCATGTCATCTACCATCA CAC	ATGGAGAAGATGGAATA CTTGC	59	156

RM212	CCACTTTCAGCTACTACC AG	CACCCATTTGTCTCTCATT ATG	54.5	136
RM495	AATCCAAGGTGCAGAGA TGG	CAACGATGACGAACACA ACC	59	159
RM10346	GCTTGATCTGCCCTTGTT TCTTGG	AACTCGAGCGGCCTTCTC AGC	65	292
RM9	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC	59	136
RM3340	TCTTGGCAAGCTCTCCTC TC	CCATCATCTCGATCTTGA CG	59	117
RM166	GGTCCTGGGTCAATAATT GGGTTACC	TTGCTGCATGATCCTAAA CCGG	59	321
RM208	TCTGCAAGCCTTGTCTGA TG	TAAGTCGATCATTGTGTG GACC	59	173
RM251	GAATGGCAATGGCGCTA G	ATGCGGTTCAAGATTCGA TC	59	147
RM85	CCAAAGATGAAACCTGG ATTG	GCACAAGGTGAGCAGTC C	59	107
RM3586	GAAGAGAGAGCCAGAGC CAG	ACACGATCGAGCTAGAA GACG	59	118
RM280	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAG G	60	155
RM252	TTCGCTGACGTGATAGGT TG	ATGACTTGATCCCGAGAA CG	59	216
RM518	CTCTTCACTCACTCACCA TGG	ATCCATCTGGAGCAAGCA AC	59	171
RM169	TGGCTGGCTCCGTGGGTA GCTG	TCCCGTTGCCGTTCATCC CTCC	71	167

RM163	ATCCATGTGCGCCTTTATGAGGA	CGCTACCTCCTTCACTTACTAGT	59	124
RM164	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	60	246
RM13	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	59	141
RM6836	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	59.5	240
RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	58.5	140
RM755	AAAGGATAAATGTGGGGATC	ATAACCGTCTGGTTTCACTG	54.5	141
RM336	CTTACAGAGAAACGGCATCG	GCTGGTTTGTTTCAGGTTCG	58	154
RM447	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC	58	111
RM256	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC	58	127
RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	52.5	225
RM201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	54.5	158
RM6100	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC	58	144
RM254	AGCCCCGAATAAATCCACCT	CTGGAGGAGCATTTGGTAGC	60	165

RM552	CGCAGTTGTGGATTTCAG TG	TGCTCAACGTTTGACTGT CC	58	195
RM26212	GTCGCTCCTCTCCTCCAA TCC	GCTCGCTGCTTCTAATCT CTTGC	62.5	180
RM3701	GAGCTAGAGGGAGGAGG TGC	TTGACTGATAGCCGATTG GG	62.5	174
RM224	ATCGATCGATCTTCACGA GG	TGCTATAAAAGGCATTCG GG	59	157
RM19	CAAAAACAGAGCAGATG AC	CTCAAGATGGACGCCAA GA	58	226
RM17	TGCCCTGTTATTTTCTTCT CTC	GGTGATCCTTTCCCATTT CA	59	184

Amplification of DNA samples with markers, separation of PCR products, and documentation of gel picture were done by following the procedures enumerated under section **3.6 & 3.7.**

3.6. Observations recorded

3.6.1. Morphological characters

The following characters were recorded on per plant basis.

1. **Plant height** (cm)**:** Distance measured from plant base to the tip of panicle excluding awn length if any and expressed in centimetre.
2. **Tillers/plant:** Number of tillers per plant was counted.
3. **Panicles/plant:** Number of panicles per plant was counted.
4. **Panicle length** (cm)**:** In each plant, panicle length of 3 panicles were measured from base to tip and the average was recorded in centimetre.
5. **Days for flowering:** Number of days taken by each plant for flowering from the date of sowing was recorded.

6. **Filled grains/panicle:** Number of filled grains in each panicle was counted for every plant and the average was recorded.
7. **Sterile grains/panicle:** Number of sterile grains in each panicle was counted for every plant and the average was recorded.
8. **Spikelet fertility** (%)**:** For each plant, spikelet fertility (%) was calculated as follows

$$\frac{\text{No. of filled grains per panicle}}{\text{Total number of grains / panicles}} \times 100$$

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 /plant** (g)**:** Weight of total grains harvested from each plant was recorded and expressed in grams.

3.6.2. Quantity and quality of DNA isolated

Quantity and quality of DNA isolated was assessed using Nanodrop. The purity of DNA samples were assessed based on A_{260}/A_{280} ratio. The ratio of 1.8 to 2 indicate pure DNA. For values greater 2, RNA contamination was evident and for values less than 1.8 protein contamination was confirmed.

3.6.3. Number of amplicons

UVITECH Fire reader software gel documentation system was used to capture the image for analyzing the band pattern and to count the number of amplicons resolved by gel electrophoresis.

3.6.4 Molecular weight analysis

The analysis of molecular weight of PCR images was done by using Navigating 1D MAX software, UVITECH Cambridge. Observations were recorded in terms of base pairs (bp) by comparison with a known molecular weight marker which was run along with the gel.

3.6.5. Statistical analysis of parameters of variability

The variability in morphology of F_4 and F_5 population were estimated.

1. **Mean:** It is calculated as the ratio of sum of individual observations to the corresponding number of individuals on which the on which the observation was made.

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

where, Xi – any observation in i^{th} treatment

N – total number of observations

2. **Range:** It is expressed as the lowest and highest values of the sample observations made.

Analysis of the genomic contribution of the parents in F_4 and F_5 population based on SSR data was carried out by using Graphical Geno Typing (GGT) version 2.0 (Van Berloo, 1999) software.

Results and discussion

IV. RESULTS AND DISCUSSION

Rice, a staple cereal crop in many parts of the world, has been confronted with multiple environmental stresses including high temperature, which negatively impacts the booting as well as anthesis growth stages. Manigbas *et al.* (2014) concluded that breeding heat-tolerant rice is one of the strategies to mitigate the effects of climate change, particularly in high temperature regions where majority of rice is grown.

In the major rice growing tracts of Kerala *viz.*, Palakkad, Kole, and Kuttanad, the temperature tends to rise up to 39 °C or higher, especially during the second/third crop. Consequently, high temperature induced spikelet sterility has become a serious problem. In order to tackle this problem, efforts were taken at College of Horticulture, Vellanikkara, Thrissur. The programme was aimed to impart heat tolerance into elite rice variety Uma (Mo 16) which is cultivated to a very large extent in Kerala.

BSA approach was employed for identification of SSR markers linked to the genes for heat tolerance in rice using segregating generation (F_3) generated from the cross Uma x N22. In this study, RM5749 showed polymorphism between tolerant and susceptible bulks. LOD value of 6.86 during SMA (Single Marker Analysis) for the linkage between marker and spikelet fertility indicated that RM 5749 on chromosome 4 is tightly linked to spikelet fertility trait under heat stress (Gorakh, 2017).

In the present study, seven selected lines from tolerant bulk of the F_3 plants of previous study were forwarded to F_4. lines selected lines from F_4 based on spike fertility percentage and genotyping were carried forward to raise F_5 generation. Selection of F_5 lines was also carried with the same criteria. Backcrossing to produce BC_1Fs from seven selected F_4 lines as female parents and Uma as male parent were also envisaged. The results obtained from the study has been are enumerated and discussed in this chapter.

4.1. Development of backcross populations (BC_1F_1 and BC_1F_2)

The major intention of backcross breeding programme is the transfer of one or few desirable genes into an otherwise popular elite cultivar. The expression of traits of economic importance is not only influenced by the genetic background but also to some extent by the external environment. Hence, resorting to both the genotypic and phenotypic selection, gives a better output in breeding programmes.

Backcrossing of the seven selected heat tolerant lines of F_3 (lines 12, 13, 15, 16, 31, 41, and 45) with recurrent parent Uma as male parent resulted in production BC_1F_1 seeds. However, the seedlings raised from these seeds did not survive under field conditions in all the three attempts made in three different seasons. The mature panicles of the female lines showed incomplete to no emergence from flag leaf sheath. This ultimately led to fungal infection in spikelets and poor seed set. (Plate 1.). Hence, the backcross progenies could not be evaluated. It is to be noted that reduced evaporation rate results in swelling of pollens which is a crucial mechanism of anther dehiscence. Shah *et al.* (2011) had mentioned that cultivars with covered panicles are better tolerant to high temperature because of their ability to reduce the evaporation rate from anthers - reduced the spikelet sterility. However, in the present study, incomplete emergence proved to be detrimental.

4.2. Morphological screening of F_4 population for heat tolerance

One hundred seeds from each of the selected seven lines of F_4 were sown. The 59 survived plants along with parents Uma and N22 were screened for heat tolerance (Plate 2.). Spikelet fertility is the major trait for selection of segregating population for heat tolerance and it is directly affected by high temperature stress. Morphological characters among survived F_4 progenies are presented in Appendix I.

Plate 1. Mature panicles covered inside flag leaf without proper emergence

N22

Uma

Plate 2. Parents N22 and Uma

4.2.1 Plant height (cm)

The plant height ranged from 69.5 (Plant no.12.2) to 99 cm (Plant no. 41.5) with an average height of 81.91 cm. Uma had an average height of 87.3 cm while N22 had 105 cm as average height.

4.2.2 Tillers/plant

Total number of tillers per plant varied from 7 (Plant no.41.1) to 14 (Plant no.12.2) with a mean of 8. Average number of tillers per plant in Uma was 10.3 while N22 was 13.

4.2.3 Panicles/plant

Number of panicles per plant ranged from 5 (Plant no. 12.2) to 11 (Plant no.12.4, 16.3, 31.3) with a mean of 8 in F_4. Average of the number of panicles per plant in Uma was 9 while in N22 it was 10.

4.2.4 Panicle length (cm)

Panicle length ranged from 16 (Plant no.12.2) to 23.7 cm (Plant no.41.5) with an average of 20 cm in F_4. Average panicle length in Uma was 21.5 cm while N22 was 17.9 cm.

4.2.5 Days for flowering

The days taken for appearance of first panicle ranged from 68 days (Plant no.13.2) to 85 days (Plant no. 41.9) with an average of 77.2 days in F_4. Uma took 91 days for the appearance of first panicle while N22 took 76 days.

4.2.6 Spikelets /panicle

The number of spikelets per panicle ranged from 37.8 (Plant no. 15.8) to 231 (Plant no.45.3) with an average of 121.1 in F_4. Average of number of spikelets in Uma was 186.93 while in N22 it was 110.86.

4.2.7 Filled grains/panicle

Filled grains per panicle were absent in Plant no.41.1, and 41.3. The highest number of filled grains per panicle (130.2) was recorded in Plant no.31.6. Average number of filled grains/panicle was 53.3 in F_4. Average of the number of filled grains per panicle in Uma was 102.2 and for N22 it was 102.8.

4.2.8 Sterile grains/panicle

Sterile grains per panicle ranged from 8.6 (Plant no.31.7) to 166.2 (Plant no.41.3) with an average of 45.76 in F_4. Average of the number of sterile grains per panicle in Uma was 84.73 while for N22 it was 8.06.

4.2.9 100 seed weight (g)

Average 100 seed weight in F_4 was 1.79 g. The trait ranged between 0 g (Plant no.41.1, 41.3) to 3.71 g (Plant no.41.11). 100 seed weight of Uma and N22 was 2.51 g and 3.9 g respectively.

4.2.10 Grain weight /plant (g)

Average grain weight or yield per plant in the F_4 population was 4.80 g. Grain weight per plant in Uma and N22 was 5.78 g and 12.4 g respectively.

4.2.11 Spikelet fertility percentage

Spikelet fertility percentage ranged from 0 % (Plant no. 41.3) to 92.54 % (Plant no.31.2) with a mean of 64.77 %. Average spikelet fertility percentage in Uma and N22 was 55.29 and 90.13 respectively.

4.3. Evaluation of selected F_4 progenies for morphological characters, yield parameters, trait similarities with UMA and N22

Evaluation of parents with F_4 lines for targeted traits like grain yield and spikelet fertility confirmed the susceptibility of Uma for heat stress while N22 was found to be a heat tolerant variety with better performance when grown in pots during third cropping season.

Table 3. Phenotyping of F_4 plants with more than 75 % spikelet fertility

Trait	Maximum	Minimum	Mean
Plant height(cm)	99.0	73.6	81.11
Tillers/plant	12.0	7.0	8.69
Panicles/plant	11.0	7.0	8.65
Panicle length (cm)	23.7	17.0	20.53
Days for flowering	85.0	69.0	77.42
Spikelets /panicle	174.4	42.2	123.54
Filled grains/panicle	130.2	18.6	79.12
Sterile grains/panicle	36.2	8.6	18.94
l00 seed weight (g)	2.7	1.3	1..89
Grain weight /plant (g)	7.6	3.1	5.82

Spikelet fertility is the major trait for identification and selection of plants for heat tolerance. Twenty six plants of F_4, showing more than 75 % spikelet fertility were selected. In this study, it was observed that heat tolerant parent N22 exhibited the highest mean spikelet fertility (90.13 %) and Uma exhibited a lower mean spikelet fertility (55.29 %). Days taken for appearance of first panicle in selected F_4 plants was equivalent to that of N22 (76 days). Filled grains per panicle in both the parents was almost similar. But, mean filled grains per panicle in the selected F_4 plants (79.12) was lesser than both parents. Mean grain weight per plant of the selected plants of F_4 was 5.82 g which is comparable to Uma. Four F_4 plants (Plant nos.16.1, 31.2, 31.3, and 31.5) with more than 90 percent spikelet fertility and 22 F_4 plants with more than 75 percent spikelet fertility were genotyped for further selection.

Table 4. IRRI Spikelet fertility classification

Spikelet fertility %	Scoring	Class
0	Completely sterile	1
1-49	Highly sterile	2
50-74	Partial sterile	3
75-90	Fertile	4
90-100	Highly fertile	5

Even though four lines exhibited more than 90 % spikelet fertility, partially filled grains were prominent in all the 26 selected plants of F_4. These 26 lines were further screened based on genome recovery and trait similarities with Uma. The F_4 lines had some undesirable traits like relatively small-sized grains and seedlings were with weak and long stems as that of N22. Both parents and F_4 plants were highly susceptible to brown plant hopper. Fungal infection was also observed in spikelets of F_4 plants. One percent Bavistin was sprayed against fungal infection. F_4 plants were severely affected with bacterial leaf blight during seedling stage. Pseudomonas at seedling stage and antibiotic Cyclin-50 at 0.6 mg/l were sprayed for controlling bacterial leaf blight in F_4 plants.

4.4. Genotyping of F_4 plants

4.4.1. Quantity and quality analysis of genomic DNA of parents and F_4 plants

Quantity and quality for the genomic DNA of F_4 plants and parents were analyzed. The A_{260}/A_{280} ratio of genomic DNA isolated from F_4 plants ranged from 2.031 to 2.1, while the average value of parents Uma and N22 ranged from 1.92 and 1.93 respectively. The results indicated that the genomic DNA extracted from the parents as well as F_4 population were of good quality as the A_{260}/A_{280} ratio of DNA extracts was between 1.8 and 2.0. A value less than 1.8 or greater than 2.0 would have indicated a high degree of protein contamination and RNase respectively (Manchester, 1996).

The quantity of the DNA isolated from the F_4 progenies varied from 359.86 µg/ml to 1697.55 µg/ml. In case of parents Uma and N22 the concentration of DNA extracted was 969.00 µg/ml and 924.24 µg/ml respectively. The concentration of DNA (50 µg/ml DNA) required for genotyping was obtained by dilution of the samples based on optical density (OD) values and DNA quantity (µg/ml) as per the procedure elaborated under section 3.4.4.

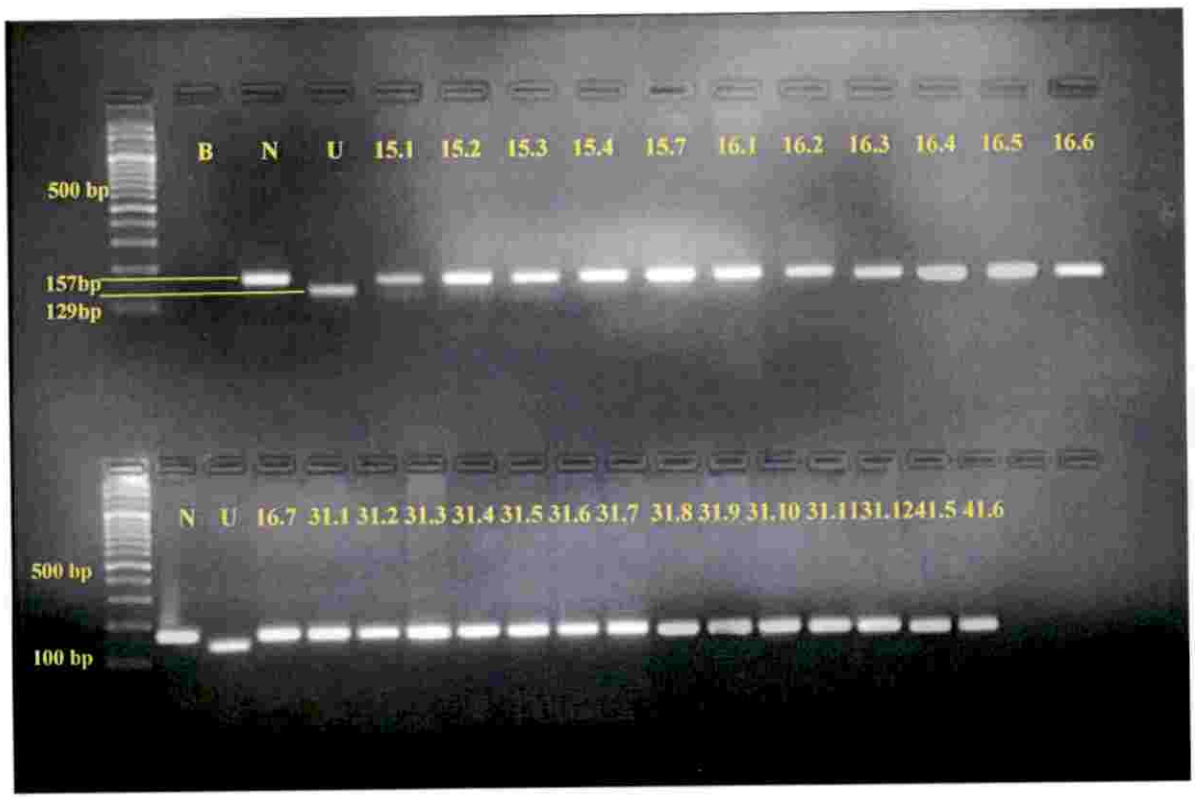
Table 5. Quality and quantity of genomic DNA of F_4 plants and parents

Genotype	Quantity of DNA (µg/ml)			Quality of DNA (A_{260}/A_{280})		
	Max	Min	Mean	Max	Min	Mean
N22 (Donor parent)	1050.20	989.20	924.24	2.05	1.86	1.93
Uma	1341.67	647.82	969.00	2.01	1.83	1.92
F_4 plants	1697.55	359.86	847.80	2.04	1.80	2.03

4.4.2. Foreground selection of F_4 population

The good quality DNA isolated from plants having high spikelet fertility (26 Nos.) and parents (N22 and Uma) were subjected to foreground selection. All the 26 F_4 plants selected based on spike fertility percentage in F_4 were genotyped using putative linked marker RM5749. Amplicons of size 157 bp and 129bp corresponding to parents N22 and Uma respectively were observed for RM5749 (Plate 3). All the 26 selected plants registered only one amplicon (157 bp) corresponding to the heat tolerant parent N22.

Gorakh (2017) reported microsatellite marker RM5749 on chromosome 4 to be tightly linked to spikelet fertility trait under heat stress and indicated that RM5749 can be used efficiently for MAS in the subsequent generations of the cross Uma x N22. The position of RM5749 was reported to be at 22.80 Mb on chromosome 4 (Gramene Database). According to a study conducted by Buu *et al.* (2014), among 264 polymorphic SSR markers between OM5930 and N22, the markers linked to high temperature stress tolerance were mostly located on chromosomes 11, 10, 8, 6, 4 and 3. RM5749 was identified linked to heat tolerance in BC_1F_1 lines obtained from a cross of OM 5930 x N22 in the same study.



N: Nagina22 **U: Uma** **B: Blank**

Plate 3 . Foreground selection of 26 F_4 plants using RM5749

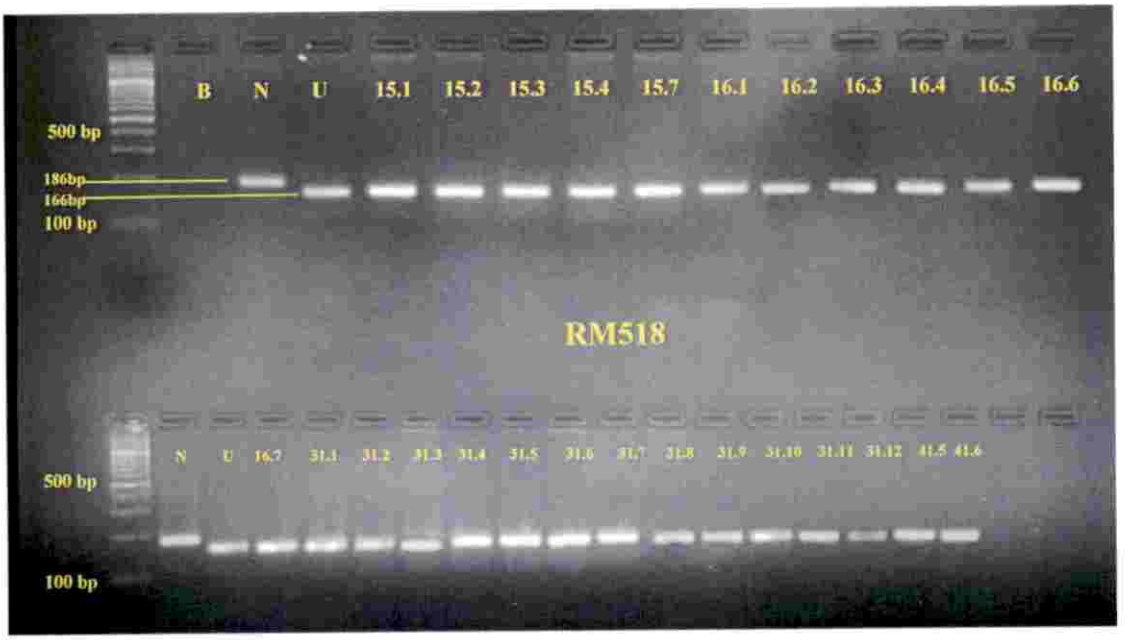
4.4.3. Allele distribution in the selected F_4 plants

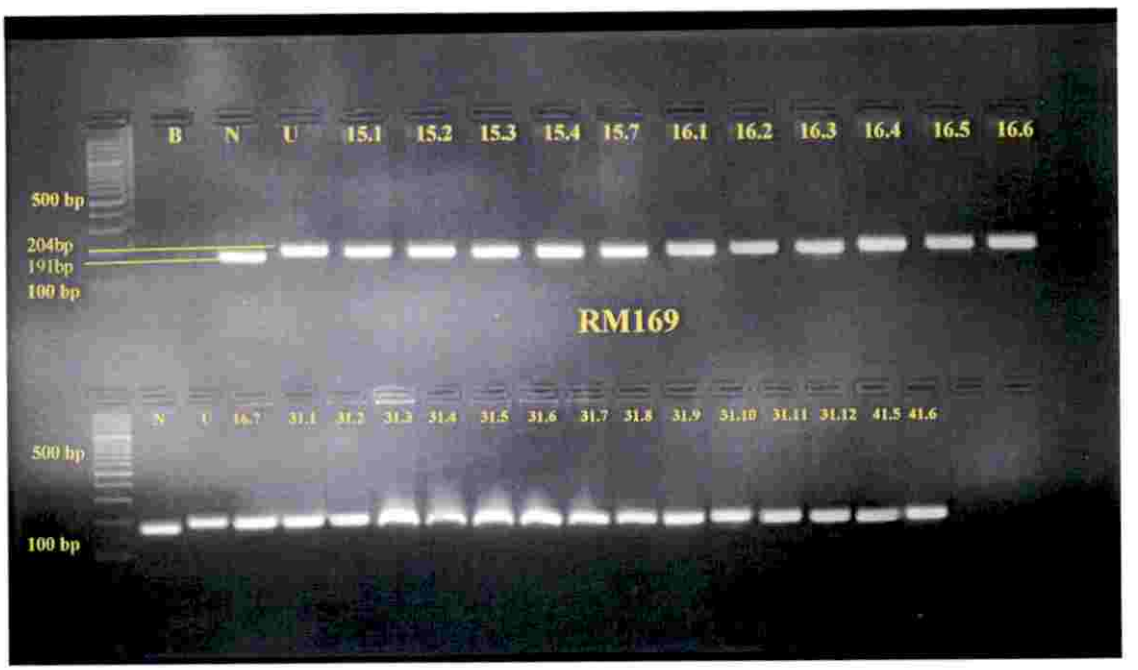
The study aimed to improve and retain the agro-morphological characters including enhancing the heat resistance in elite cultivar Uma. Chukwu *et al.* (2019) opined that marker assisted selection with phenotypic screening can be combined in order to maximise the genetic gain.

Thirty-five rice microsatellite that were reported to be polymorphic between the parents, Uma and N22, were used for screening the selected 26 plants of F_4 population. The results are presented in Table 6. Out of these, l4 were recognised for their association with QTL for heat tolerance by earlier researchers. Buu *et al.* (2014) had reported RM7076, RM3586, RM26212, and RM5749 as polymorphic markers for heat tolerance between parents. As per the reports of Zhao *et al.* (2016), RM3340, RM447, RM5545, RM3701, and RM336 were polymorphic markers for heat tolerance. RM473A as identified by Liao *et al.* (2011) and RM251 as identified by Buu *et al.* (2013) were polymorphic markers for heat tolerance between parents. Poli *et al.* (2013) and Bharathkumar *et al.* (2014) respectively reported RM225 and RM6l00 as polymorphic markers for heat tolerance. RM242 is reported to be a polymorphic marker for heat tolerance (Wei *et al.,* 2013). According to Xiao *et al.* (2011), RM6100 marker is linked with a major quantitative trait locus (QTL) on chromosome 10 for heat stress tolerance at flowering stage.

Of the 35 polymorphic SSR markers used, nine markers (RM13, RM163, RM164, RM166, RM169, RM212, RM302, RM518, and RM7076) exhibited the same allelic pattern of the female parent (Uma) in 26 plants of F_4 having high spike fertility percentage indicating that the F_4 and the female parent had identical alleles at these marker loci. (Plate 4.l. to 4.5.)

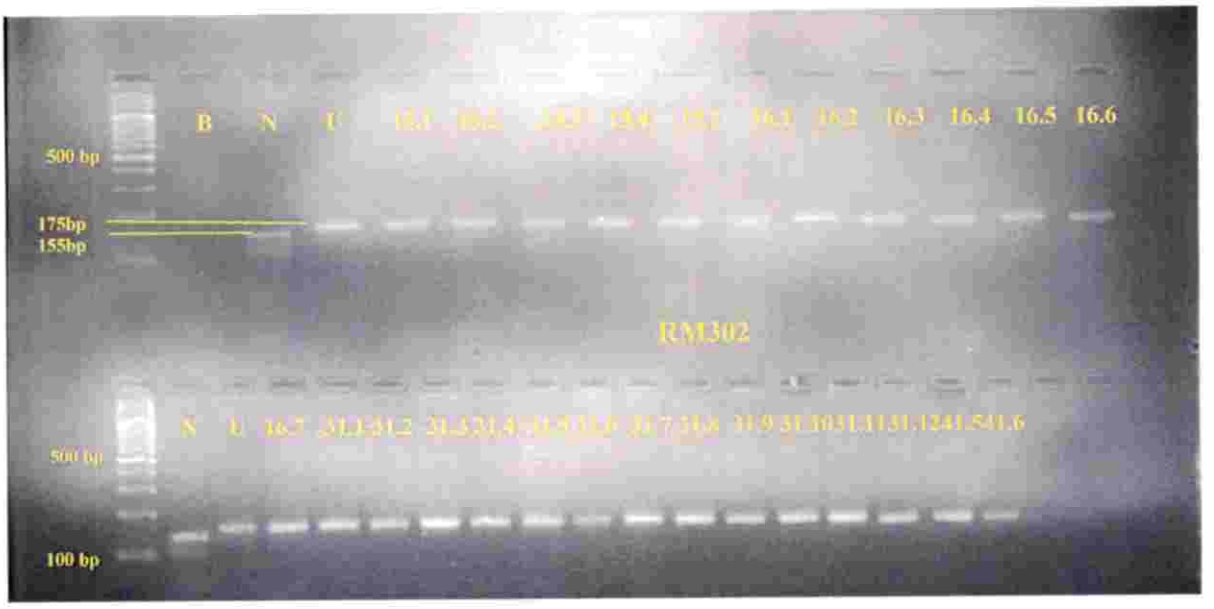
Ten markers (RM9, RM208, RM201, RM225, RM242, RM495, RM3586, RM6836, RM26212, and RM6100) exhibited the same allelic pattern of the male parent





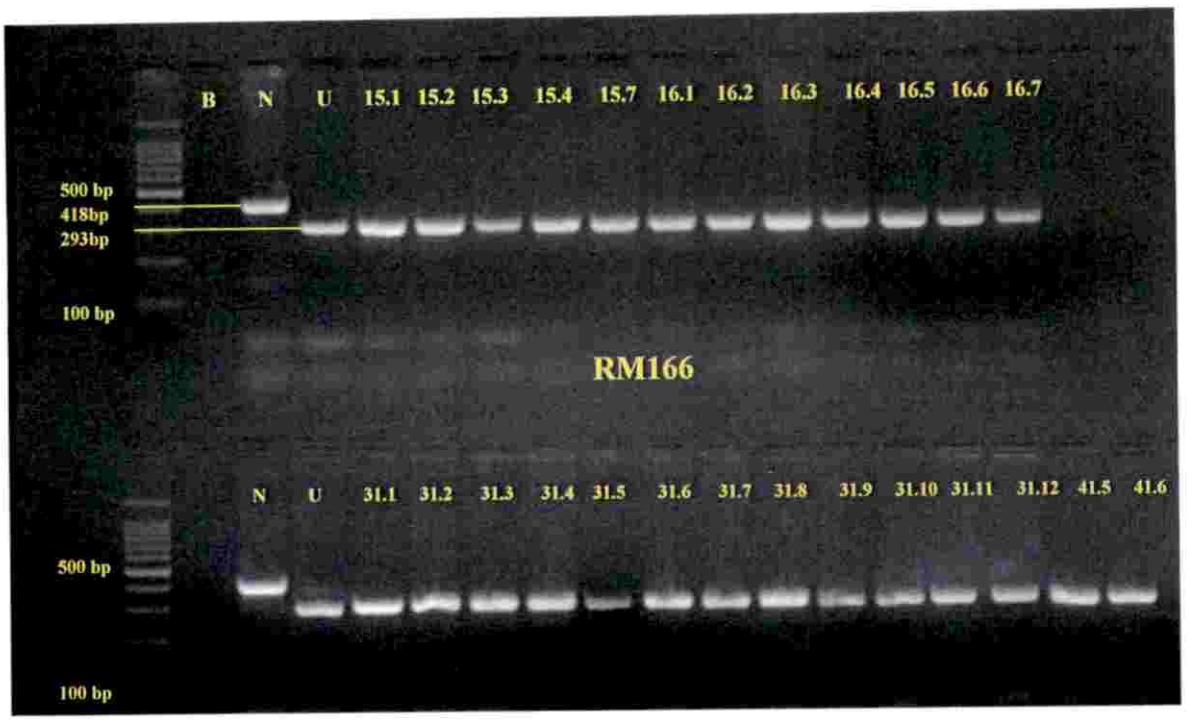
N: Nagina22 **U: Uma** **B: Blank**

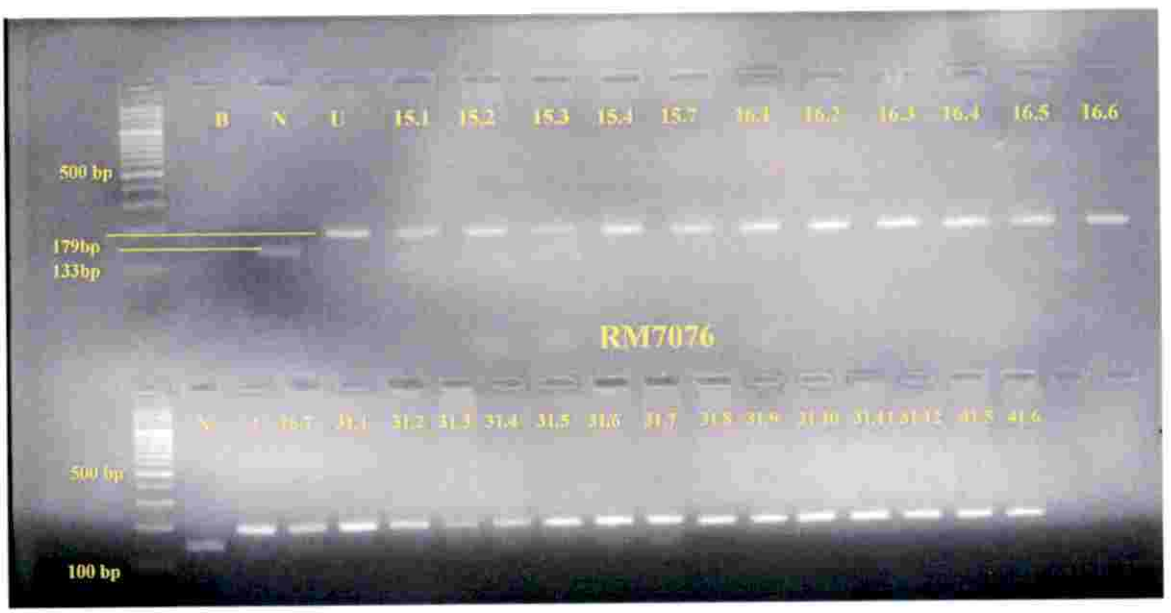
Plate 4.1. Background selection of the 26 fertile plants in F_4 using RM518 and RM169



N: Nagina22 **U: Uma** **B: Blank**

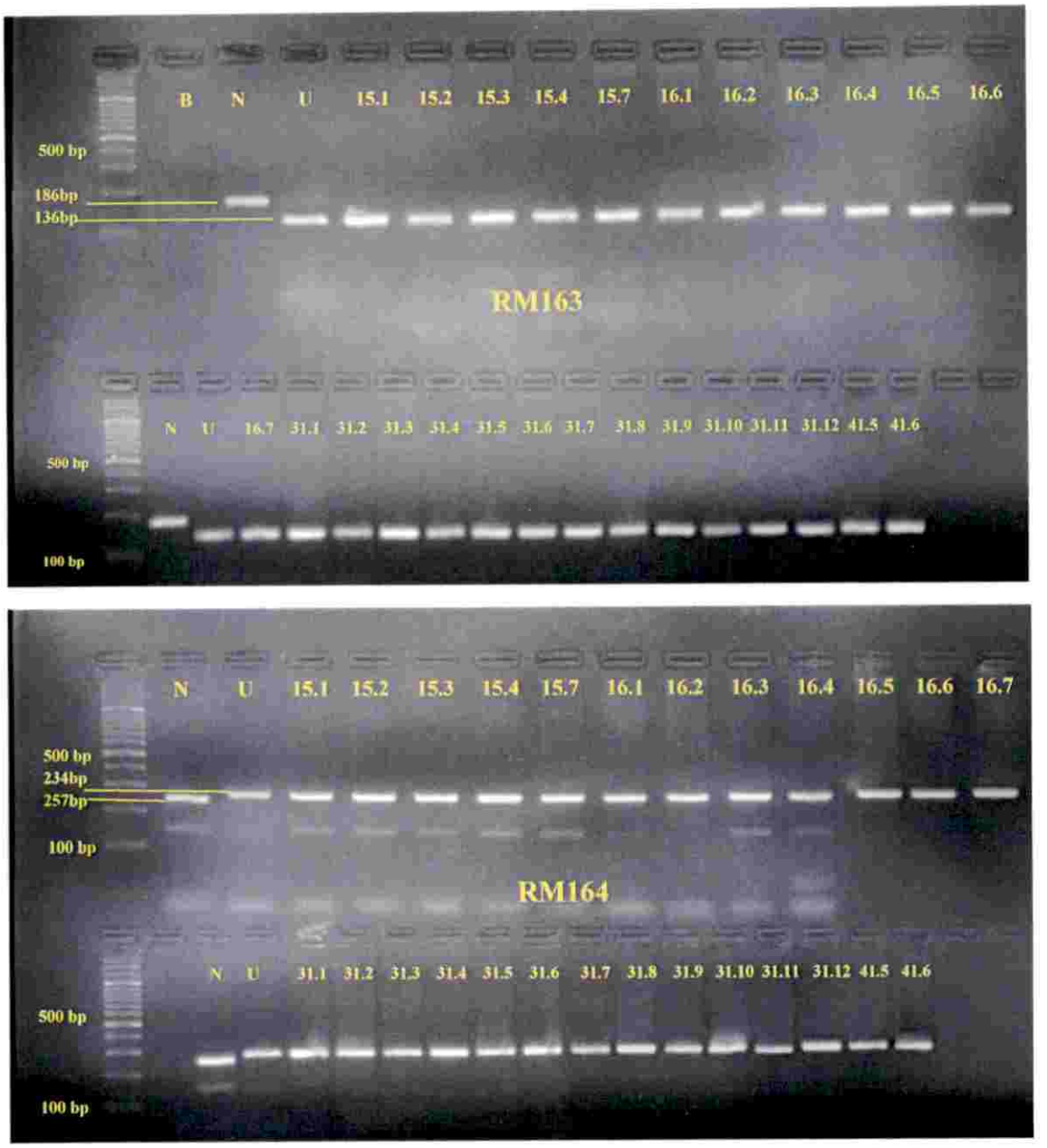
Plate 4.2. Background selection of the 26 fertile plants in F_4 using RM302 and RM212





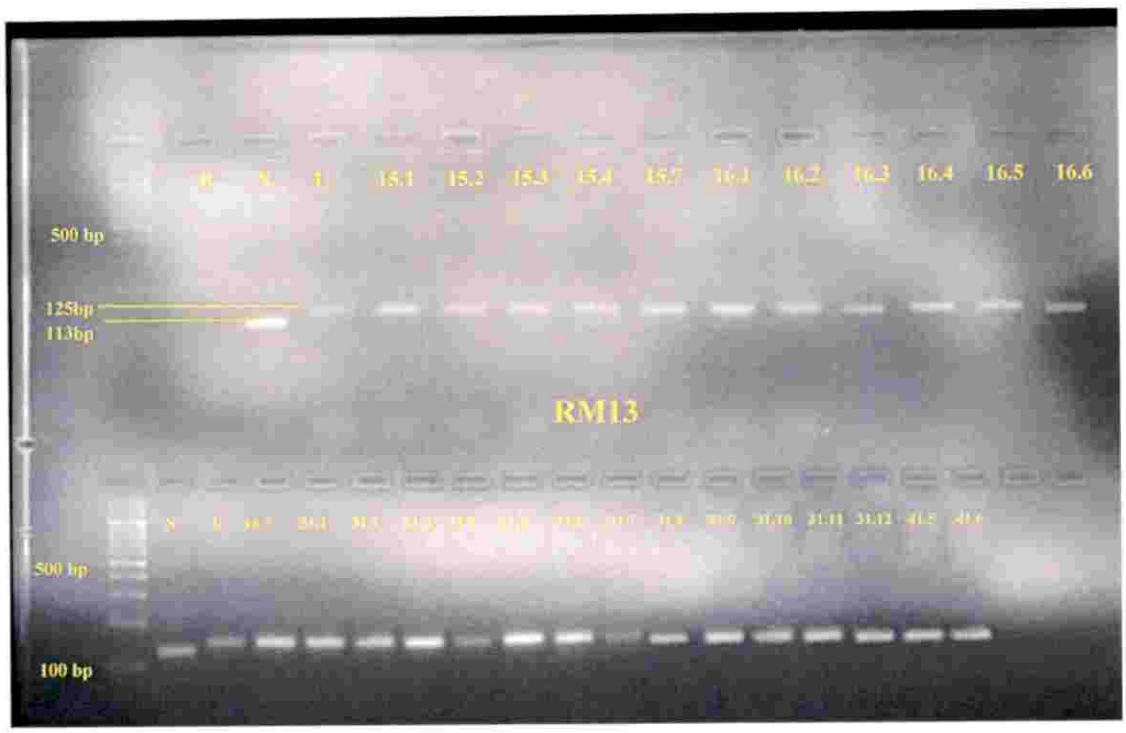
N: Nagina22 **U: Uma** **B: Blank**

Plate 4.3 Background selection of the 26 fertile plants in F_4 using RM166 and RM7076



N: Nagina22 **U: Uma** **B: Blank**

Plate 4.4. Background selection of the 26 fertile plants in F_4 using RM163 and RM164



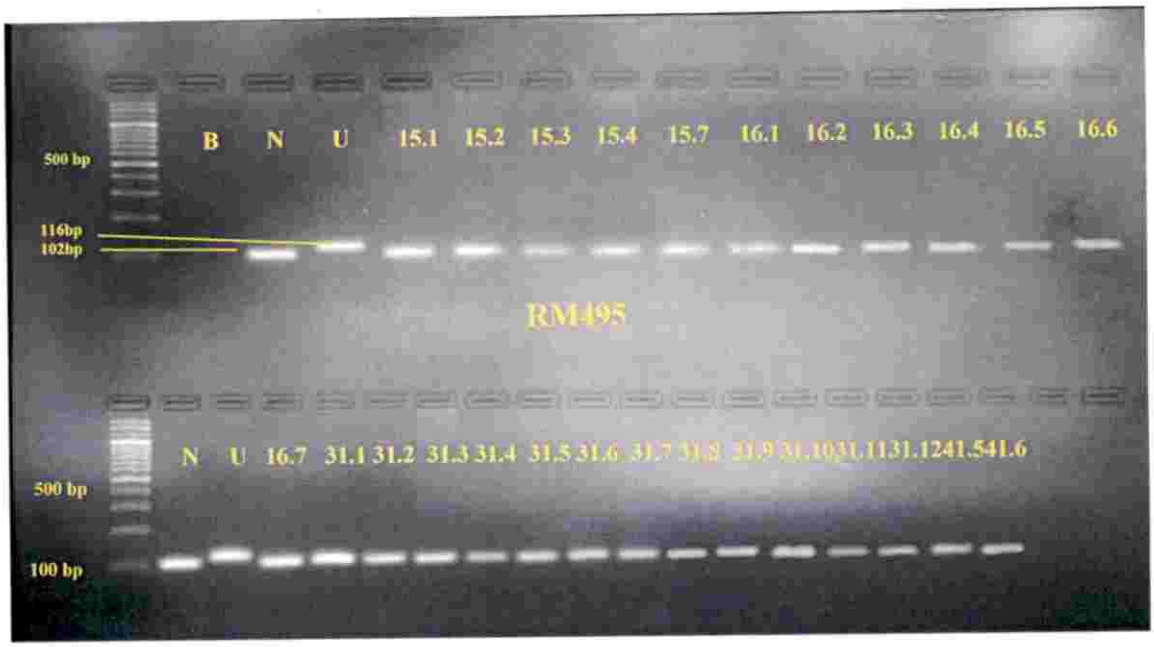
N: Nagina22 **U: Uma** **B: Blank**

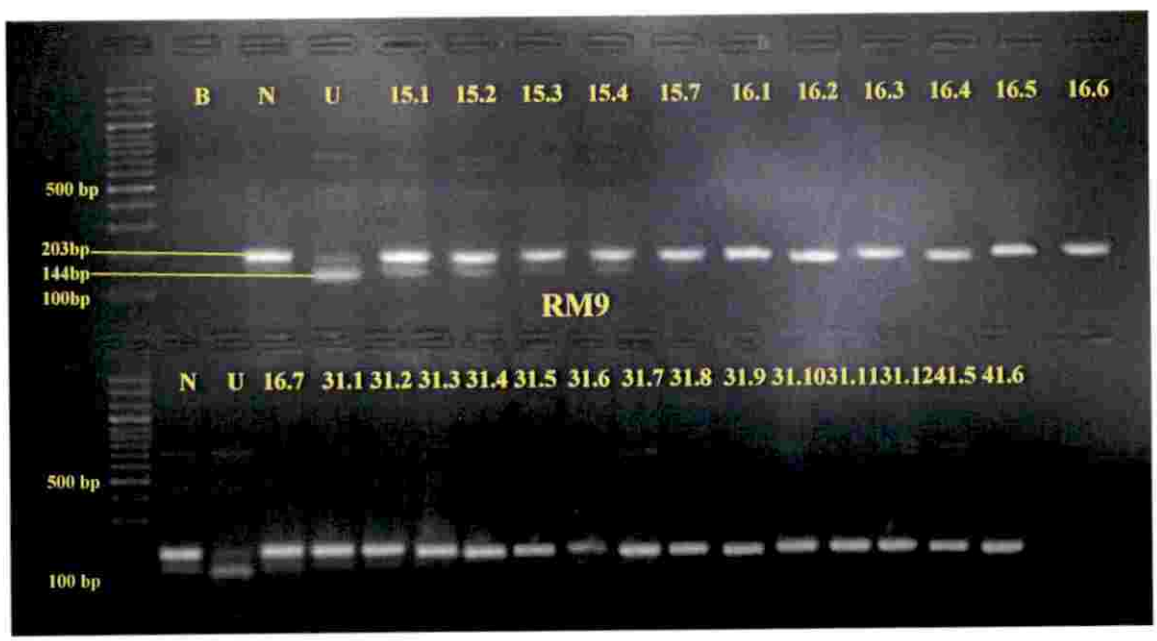
Plate 4.5. Background selection of the 26 fertile plants in F_4 using RM13

N22 for these 26 fertile plants of F_4 studied indicating the similarity in alleles with donor parent at these marker loci. (Plate 5.1. to 5.5.)

Presence of alleles of both the parents were observed in the selected F_4 Plants with respect to seventeen SSR markers. This revealed the heterozygous nature of these l6 marker loci in the selected 26 F_4 plants. (Plate 6.1. to 6.8.)

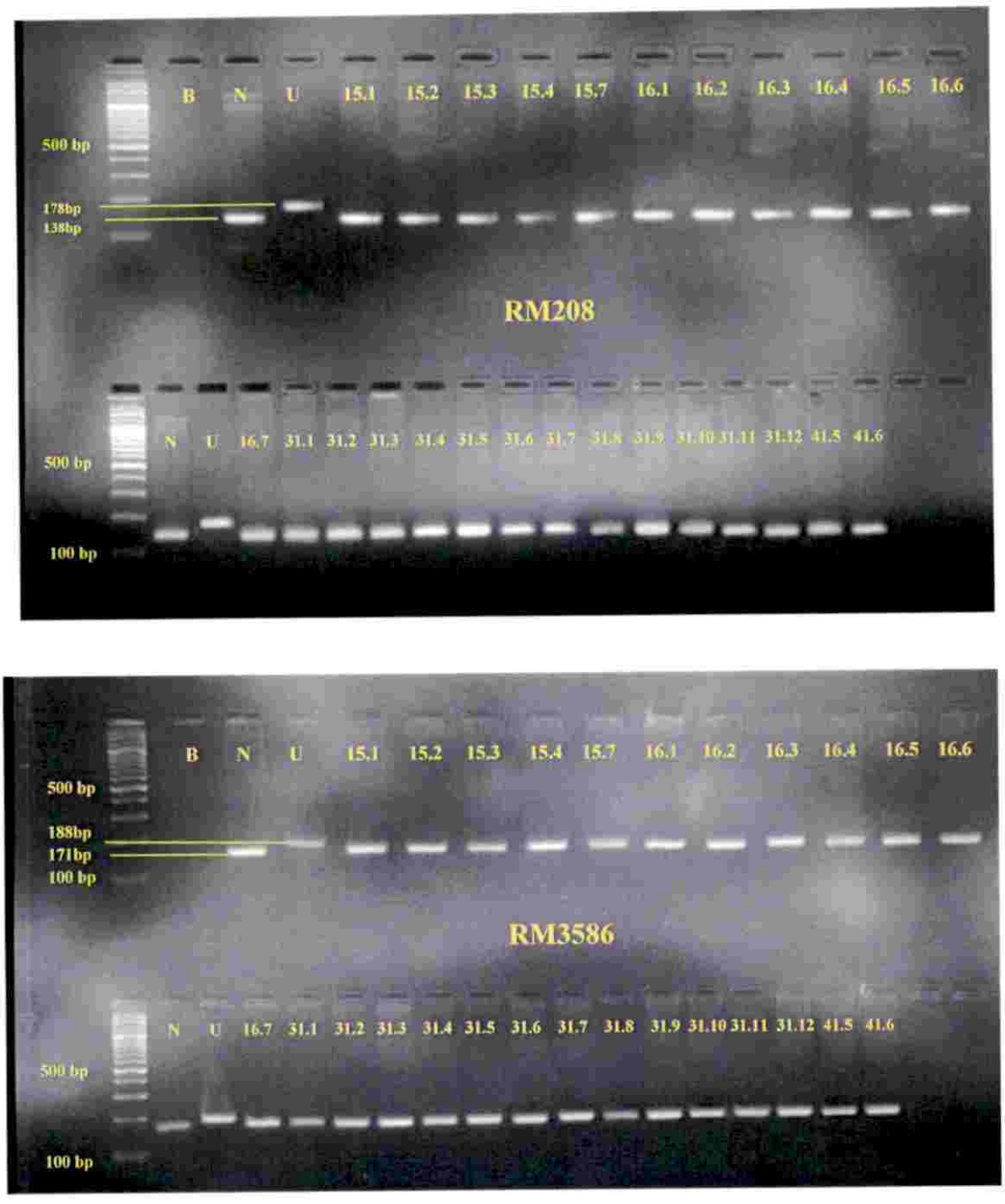
These selected 26 lines were further screened based on genome recovery using the marker data and trait similarities with Uma. Among these, the highest genome recovery of Uma was in plant Nos. 16.7. and 31.9 with 67.14 per cent and 65.71 per cent recovery respectively. Seven plants (Plant No.15.3, 31.3, 31.4, 31.5, 31.7, 31.10, and 41.5) showed 64.29 per cent recovery with Uma and two plants 15.4 and 15.7 showed 52.86 per cent and 54.29 per cent recovery of N22. The GGT software output indicated that maximum genomic regions of parent Uma were present on chromosome 3 and chromosome 5. Heterozygous loci were located on chromosomes 3, 5, 11, and 12 (Figure l).





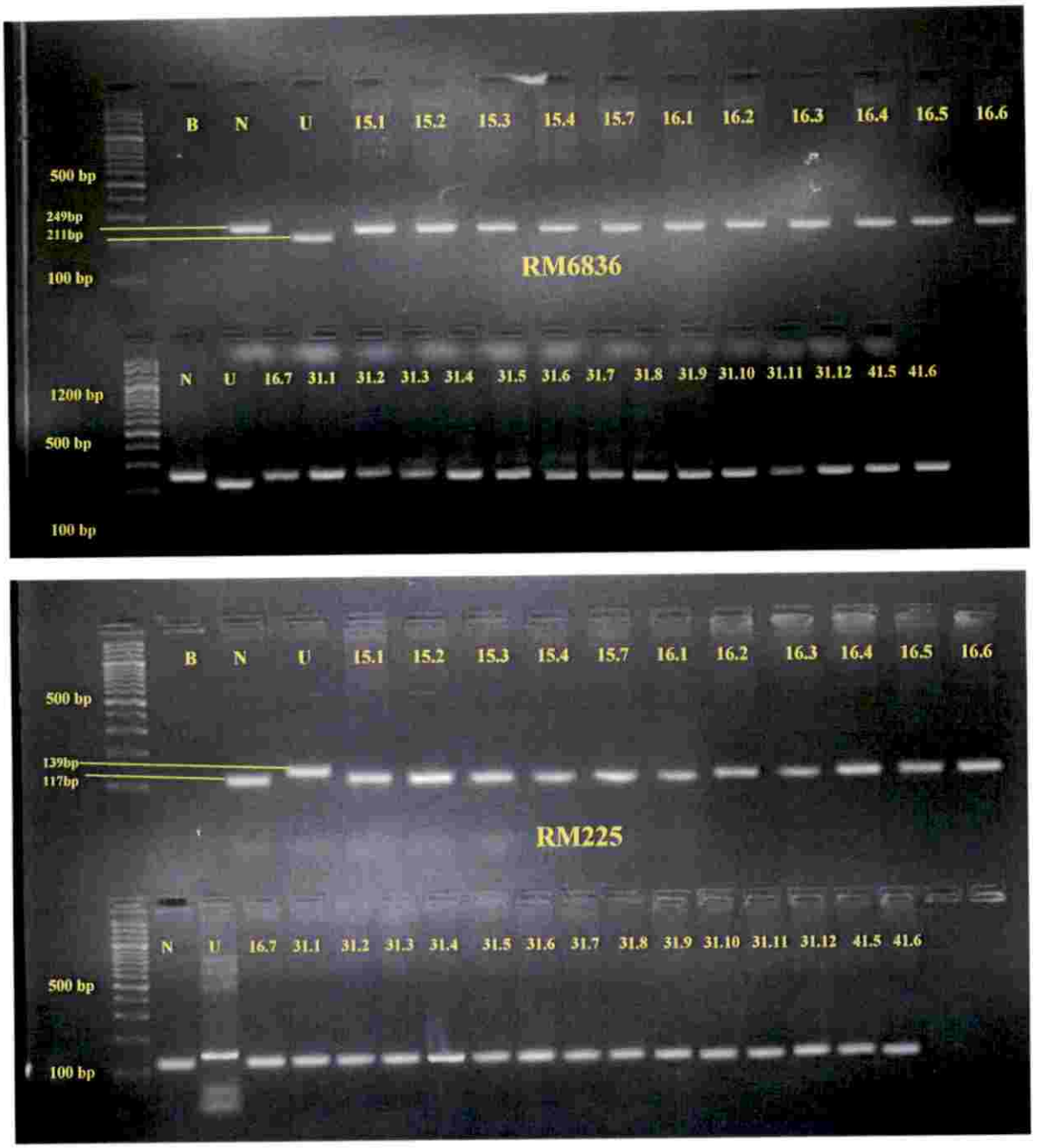
N: Nagina22 **U: Uma** **B: Blank**

Plate 5.1. Background selection of the 26 fertile plants in F_4 using RM495 and RM9



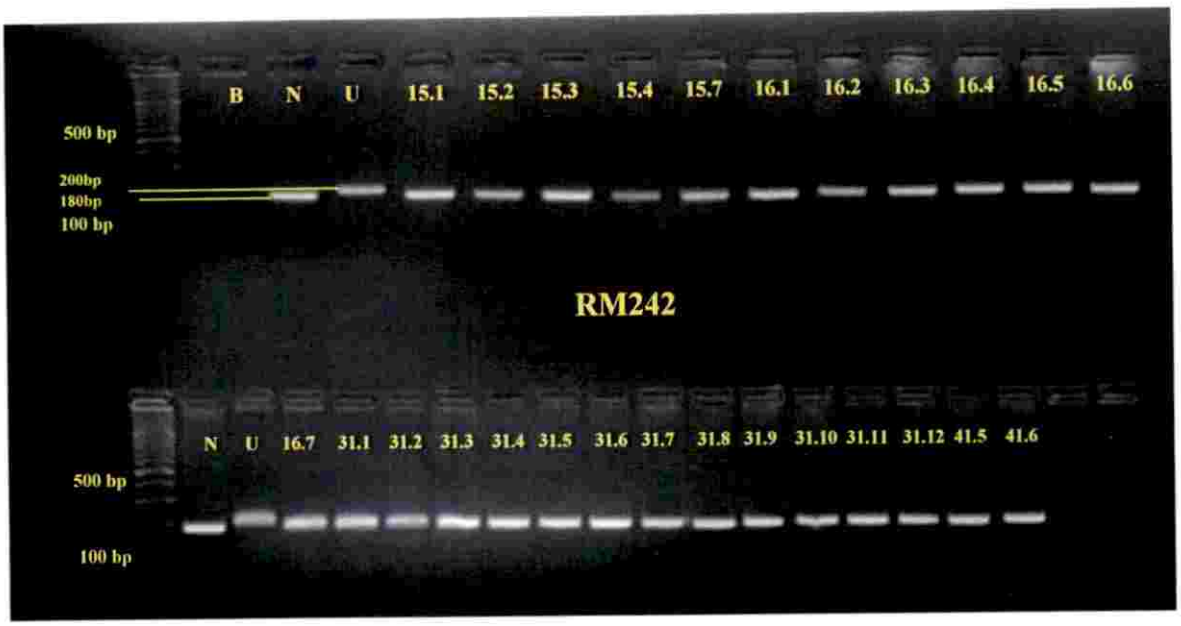
N: Nagina22 **U: Uma** **B: Blank**

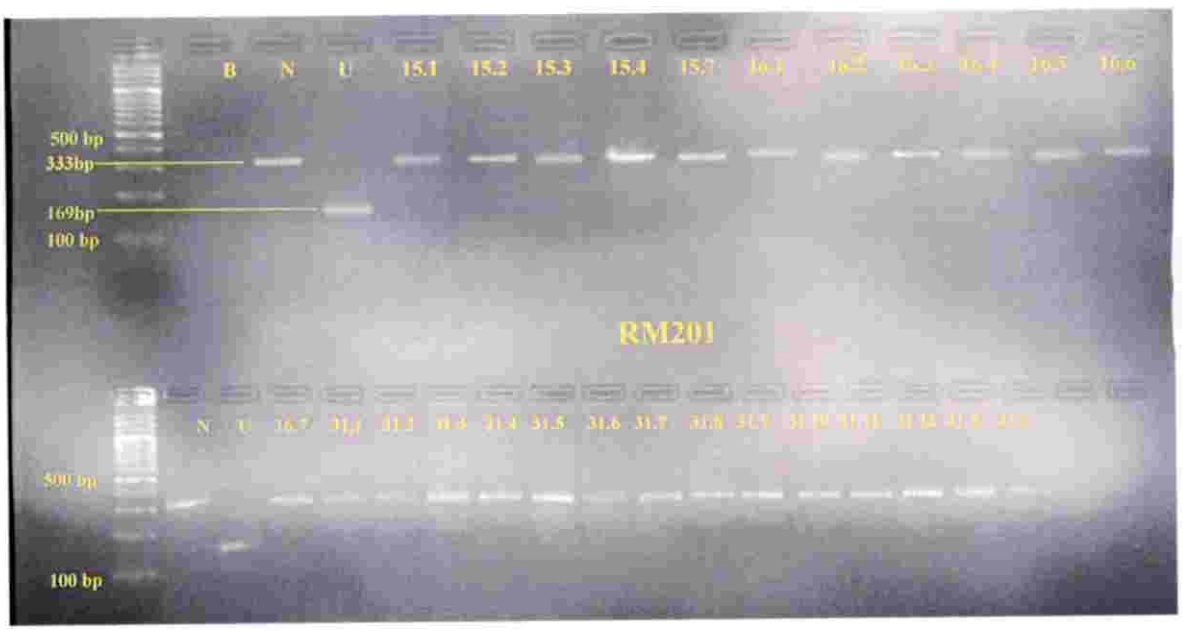
Plate 5.2. Background selection of the 26 fertile plants in F_4 using RM208 and RM3586



N: Nagina22 **U: Uma** **B: Blank**

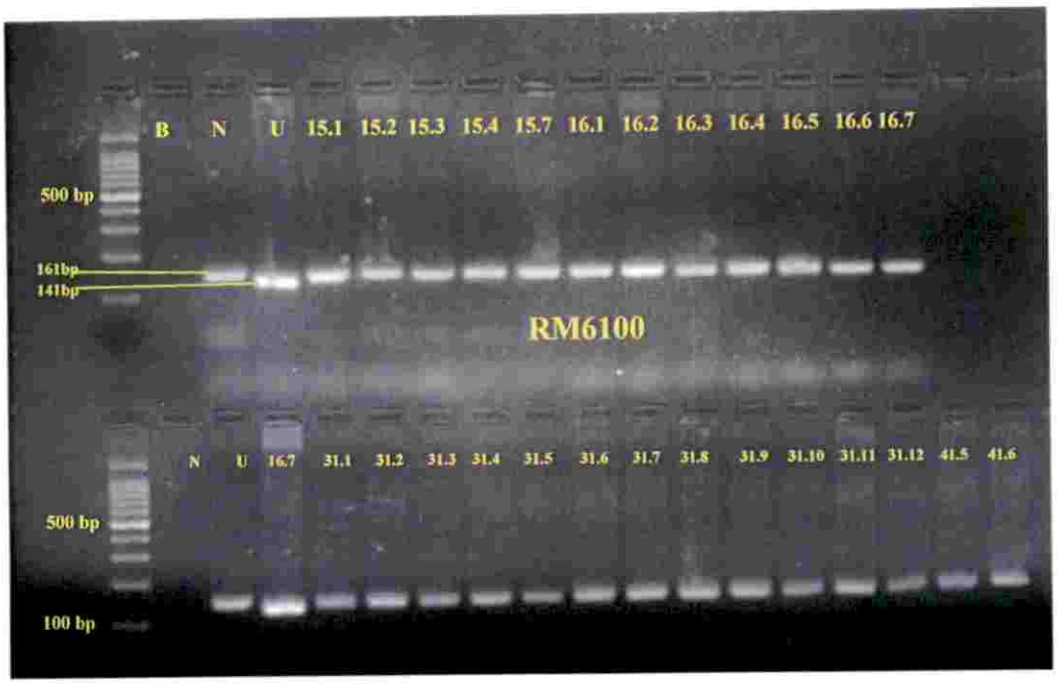
Plate 5.3. Background selection of the 26 fertile plants in F_4 using RM6836 and RM225





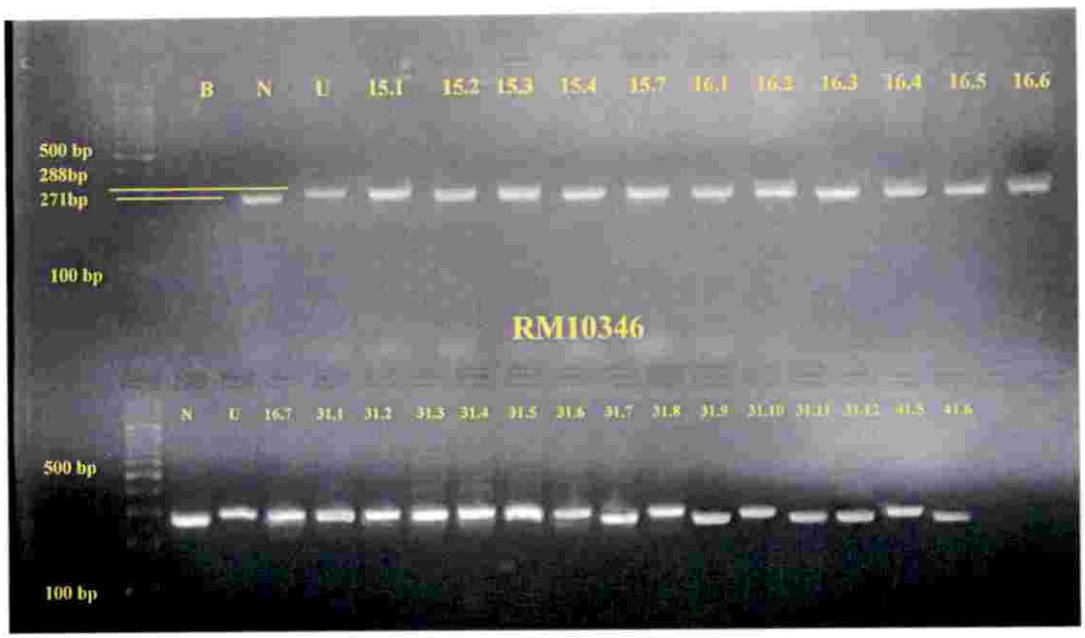
N: Nagina22 **U: Uma** **B: Blank**

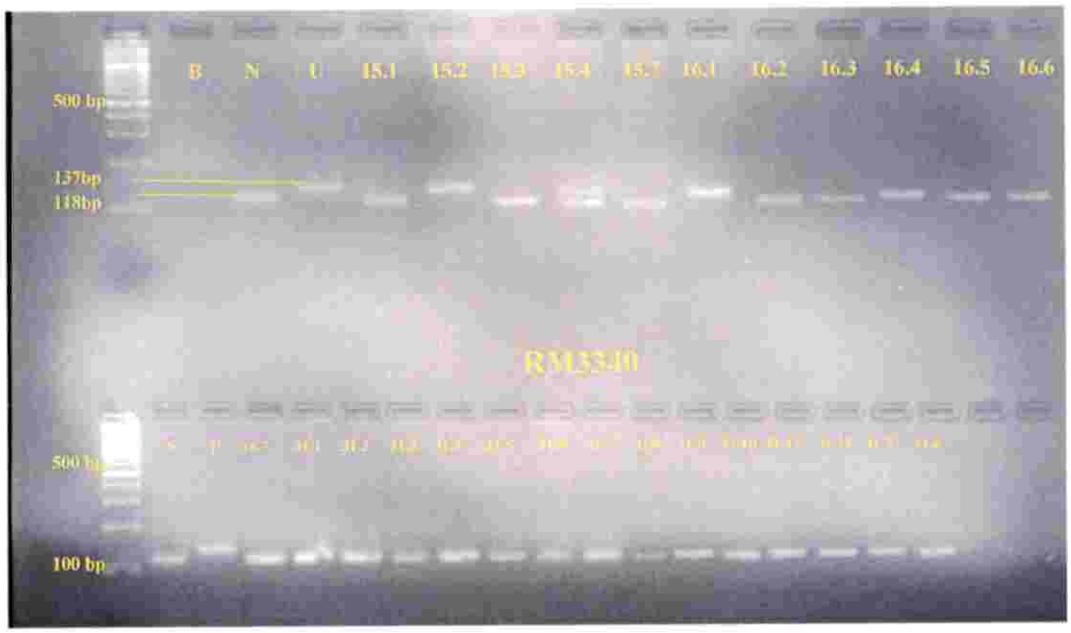
Plate 5.4. Background selection of the 26 fertile plants in F_4 using RM242 and RM201



N: Nagina22 **U: Uma** **B: Blank**

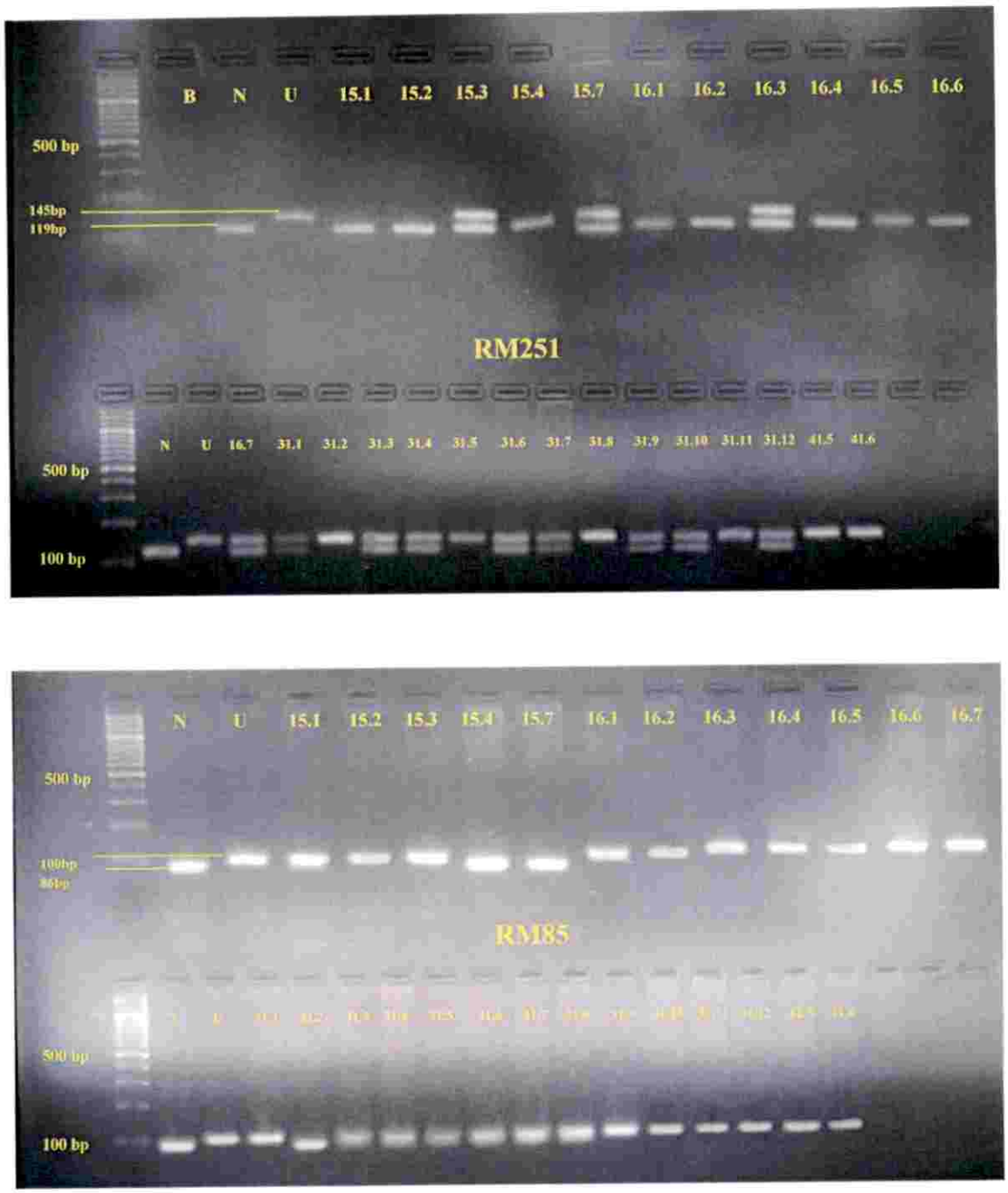
Plate 5.5. Background selection of the 26 fertile plants in F_4 using RM6100 and RM26212





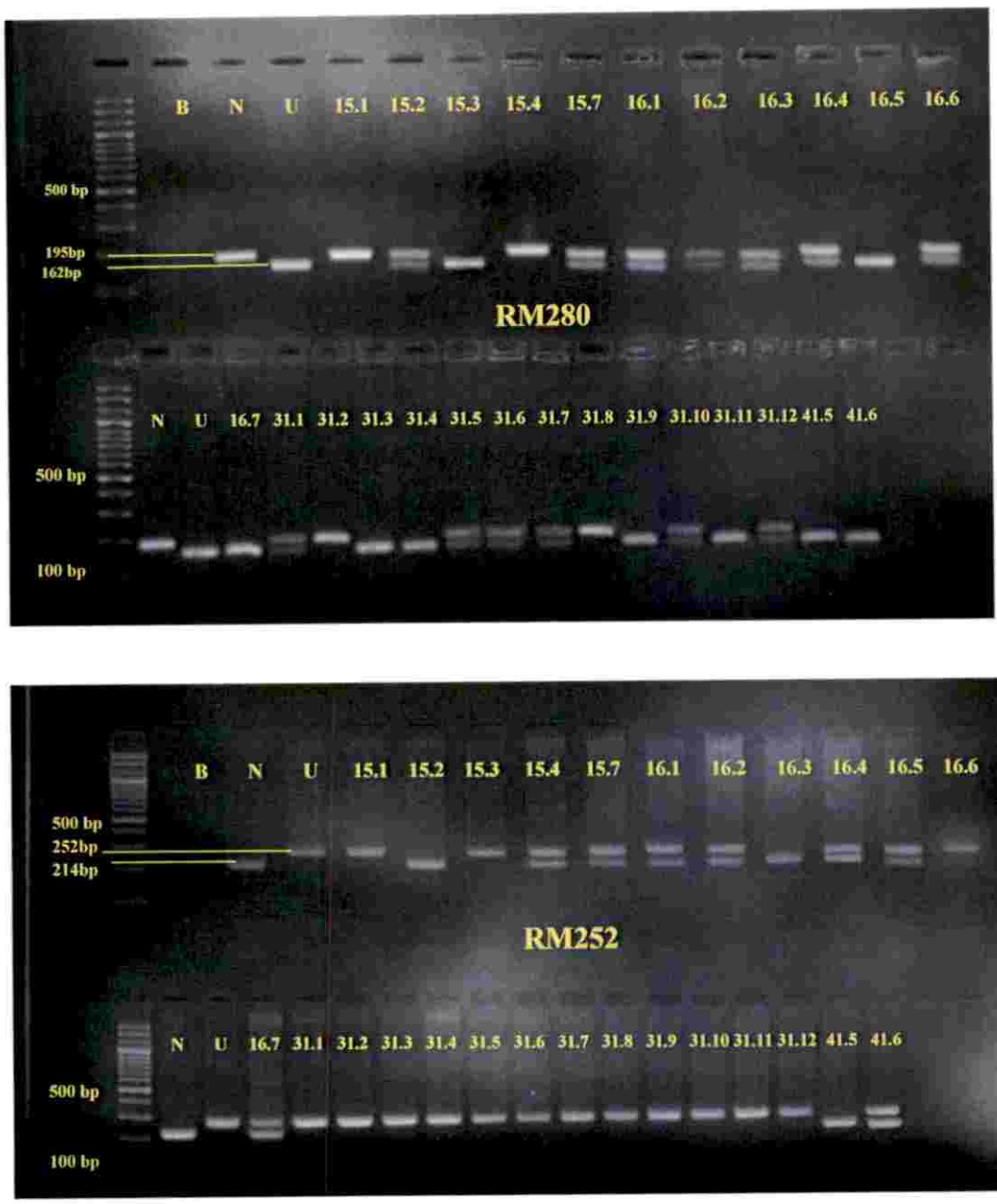
N: Nagina22 **U: Uma** **B: Blank**

Plate 6.1. Background selection of the 26 fertile plants in F_4 using RM10346 and RM3340



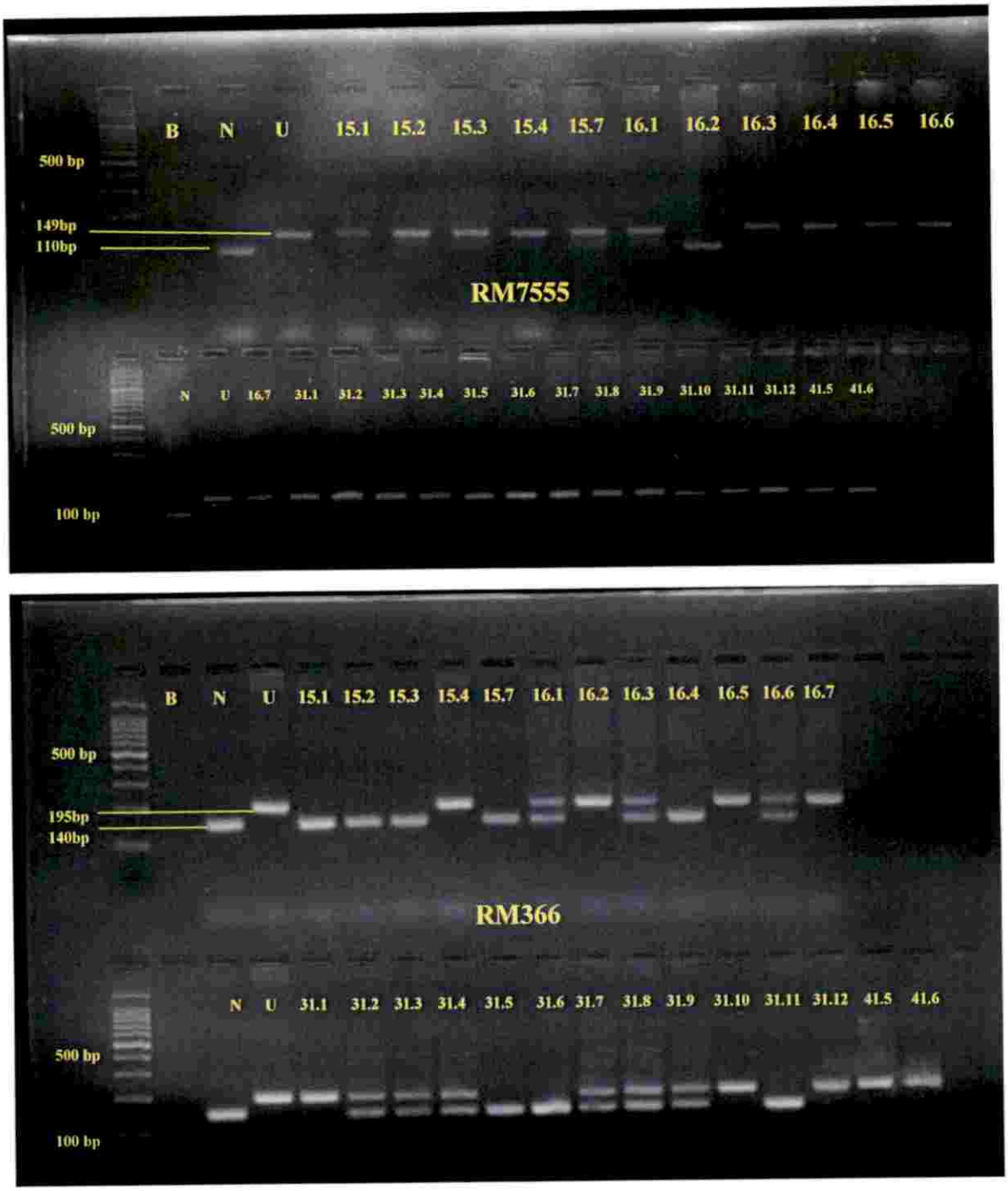
N: Nagina22 **U: Uma** **B: Blank**

Plate 6.2. Background selection of the 26 fertile plants in F_4 using RM251 and RM85



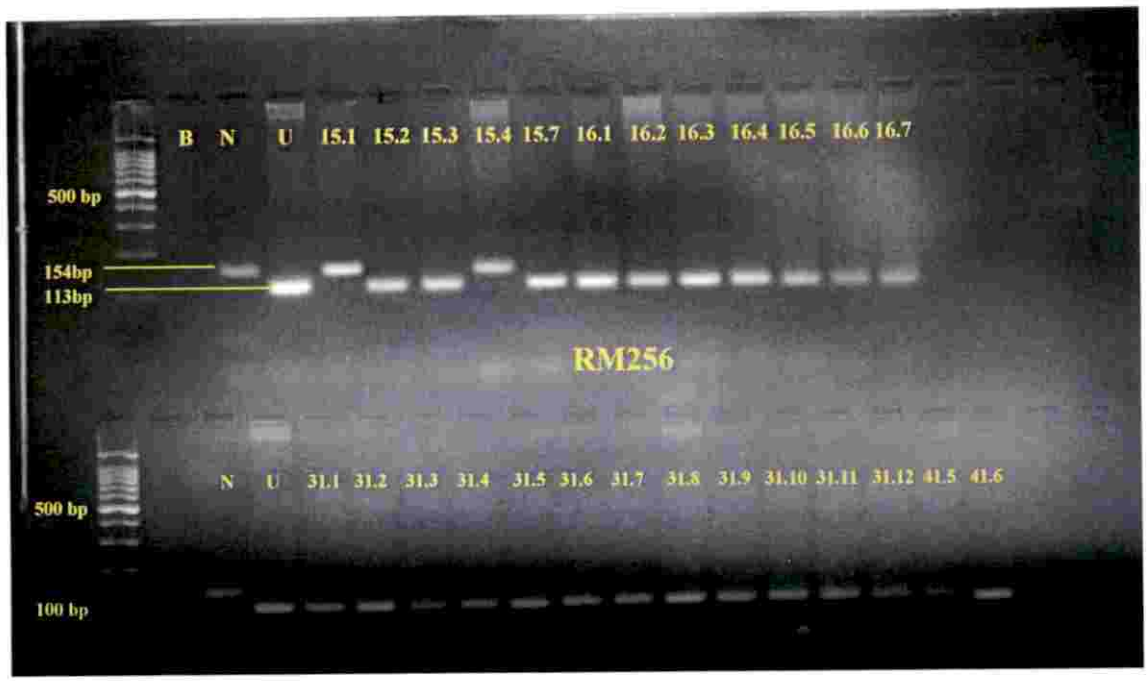
N: Nagina22 **U: Uma** **B: Blank**

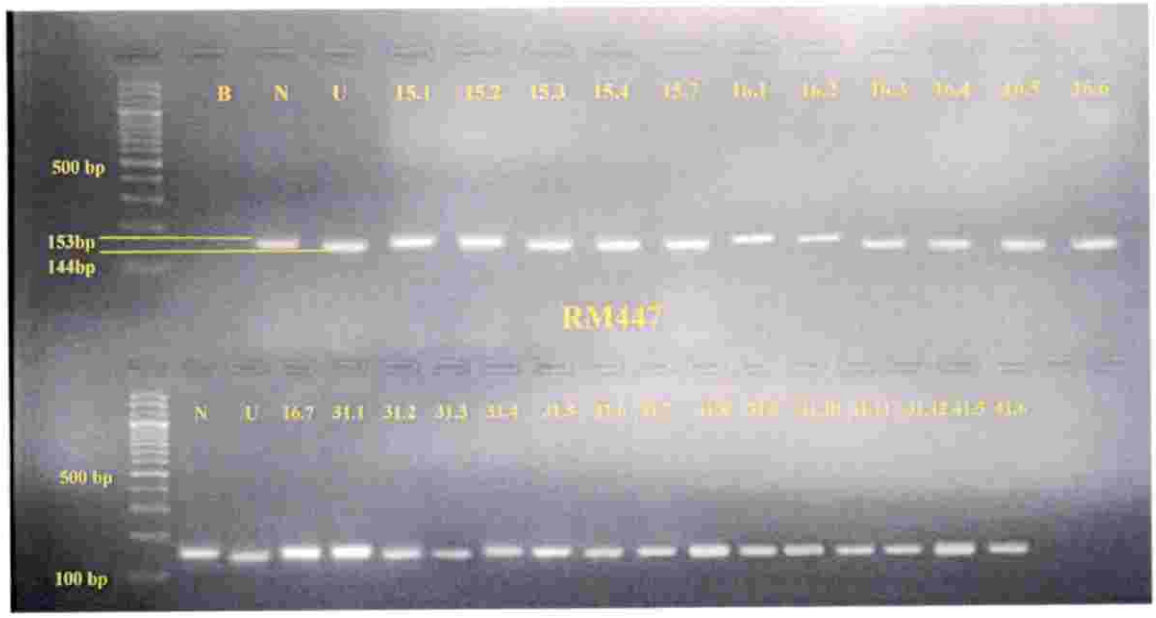
Plate 6.3. Background selection of the 26 fertile plants in F_4 using RM280 and RM252



N: Nagina22 **U: Uma** **B: Blank**

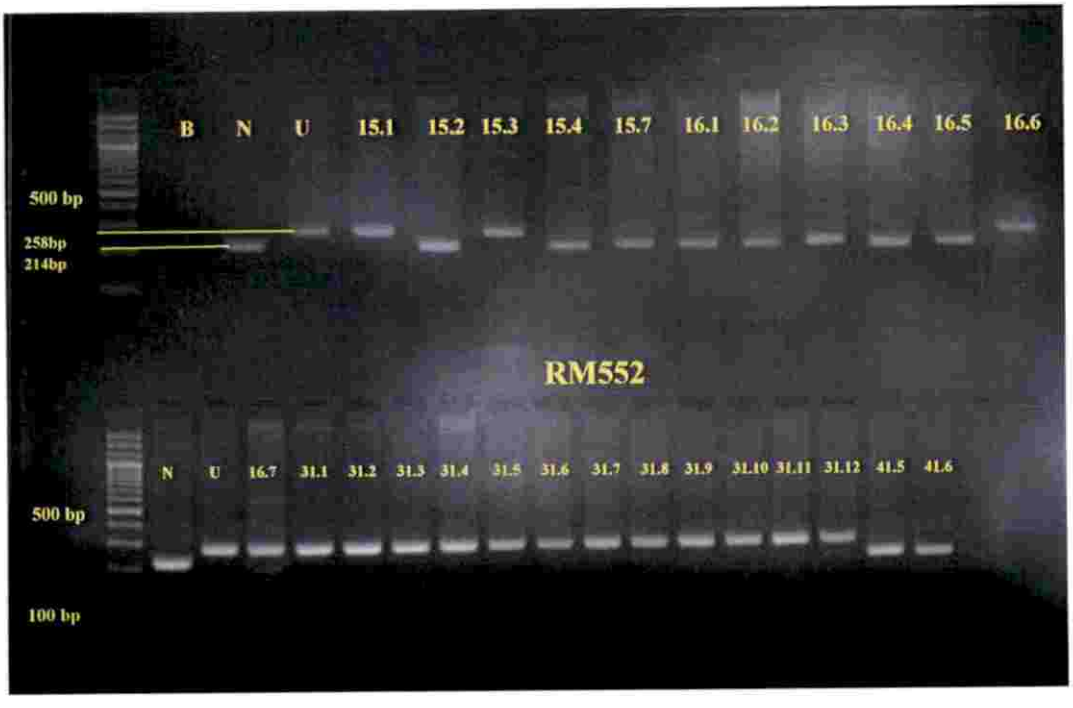
Plate 6.4. Background selection of the 26 fertile plants in F_4 using RM7555 and RM366





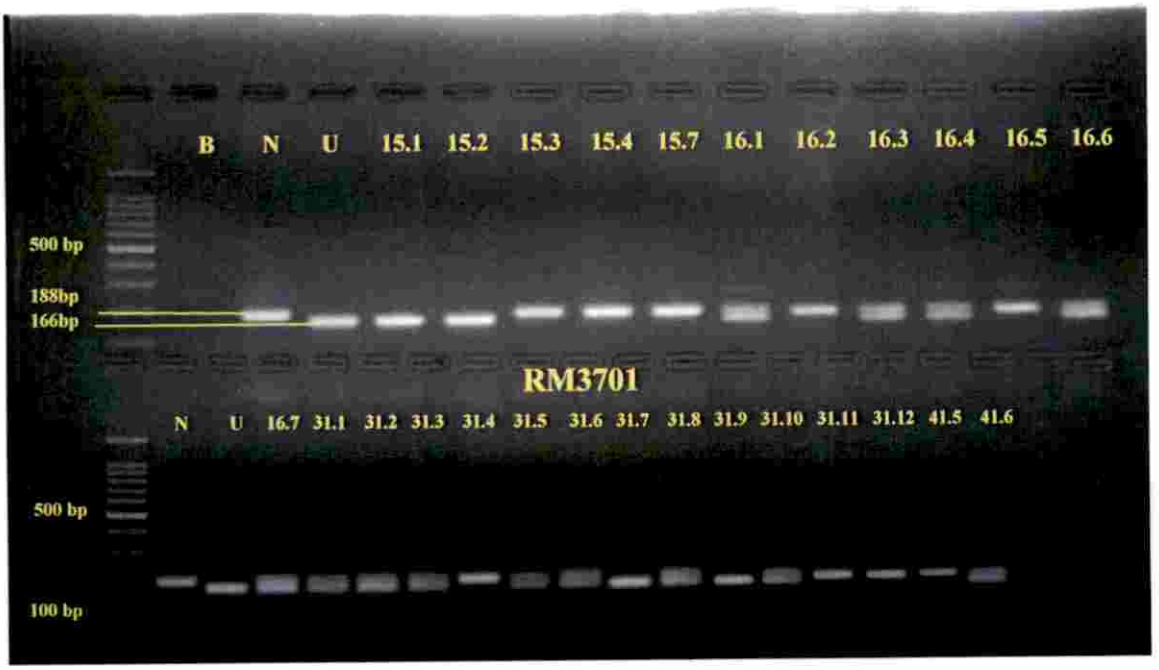
N: Nagina22 **U: Uma** **B: Blank**

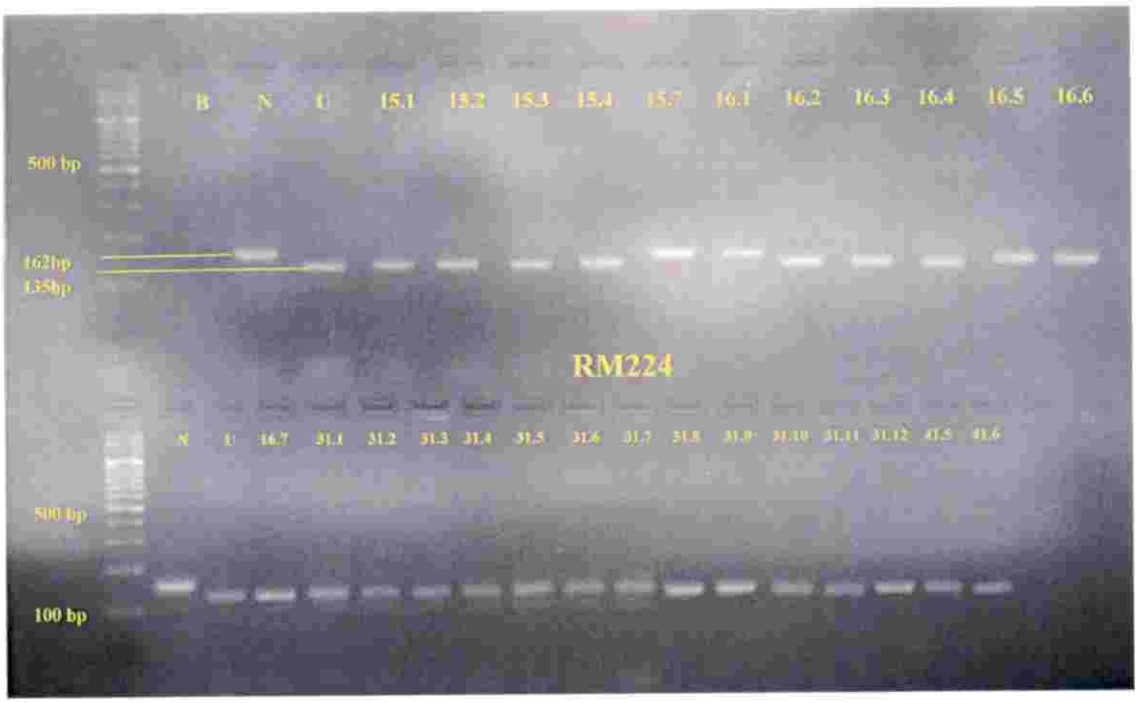
Plate 6.5. Background selection of the 26 fertile plants in F_4 using RM256 and RM447



N: Nagina22 **U: Uma** **B: Blank**

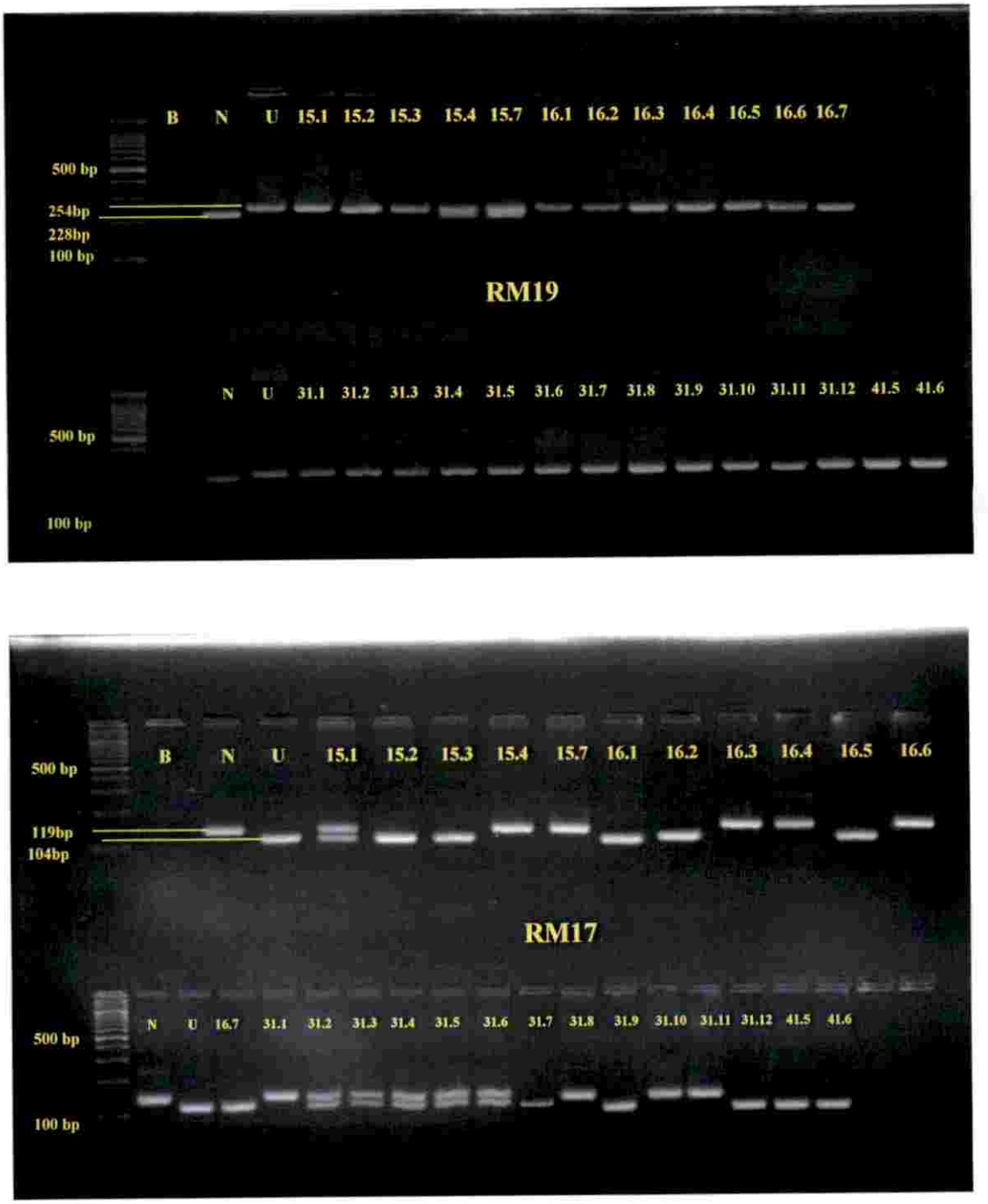
Plate 6.6. Background selection of the 26 fertile plants in F_4 using RM254 and RM552





N: Nagina22 **U: Uma** **B: Blank**

Plate 6.7. Background selection of the 26 fertile plants in F_4 using RM3701 and RM224



N: Nagina22 **U: Uma** **B: Blank**

Plate 6.8. Background selection of the 26 fertile plants in F_4 using RM19 and RM17

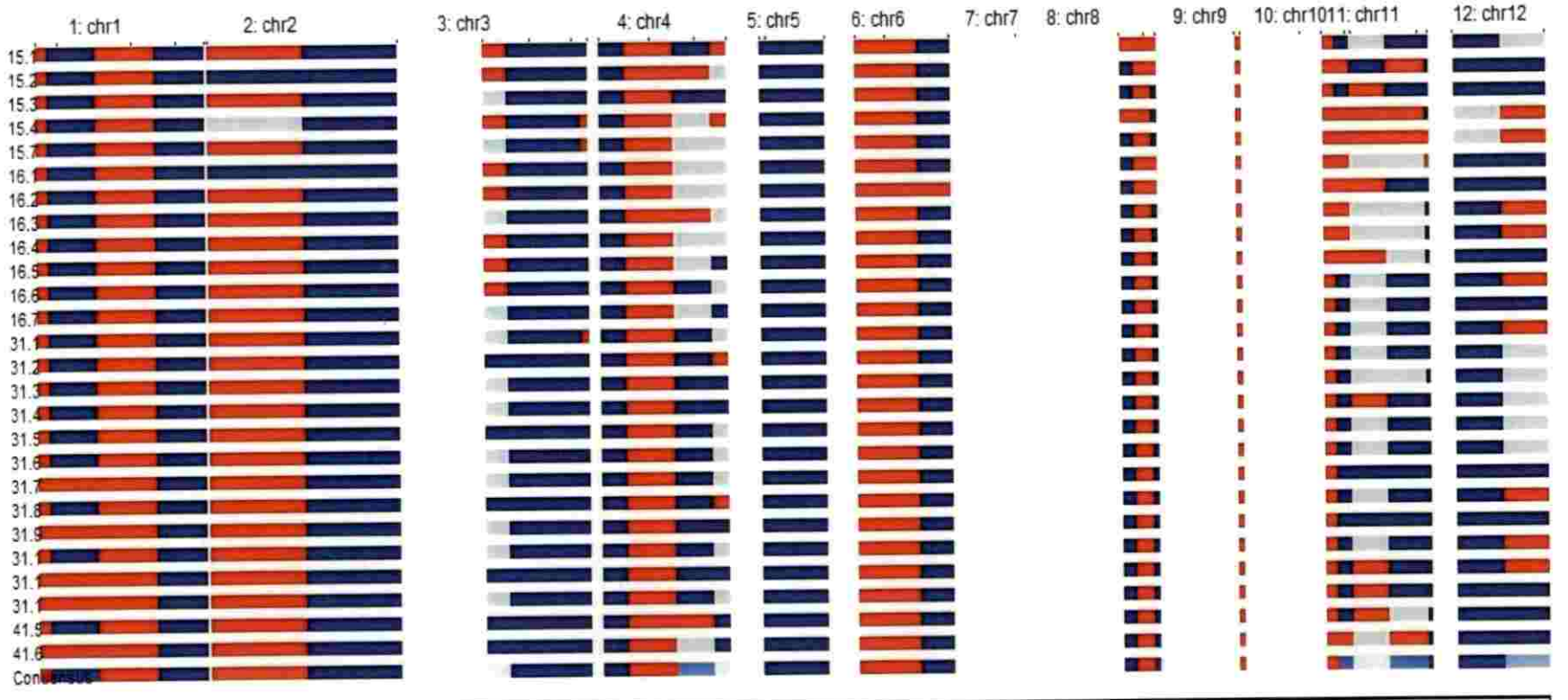


Figure. 1. GGT software output for the genome of 26 fertile plants of F_4 population

Table 6. Distribution of alleles of marker loci used for background selection in the selected F_4 plants

Sl. No	RM Marker	N22	15.1	15.2	15.3	15.4	15.7	16.1	16.2	16.3	16.4	16.5	16.6	16.7	31.1	31.2	31.3	31.4	31.5	31.6	31.7	31.8	31.9	31.10	31.11	31.12	41.5	41.6	UMA
1	RM302	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
2	RM212	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
3	RM495	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
4	RM10346	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	A	B	A	A	B	A	B
5	RM9	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
6	RM3340	A	A	B	A	H	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
7	RM166	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
8	RM251	A	A	A	H	A	H	A	A	H	A	A	A	H	H	B	H	H	B	H	H	B	H	H	B	H	B	B	B
9	RM208	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
10	RM85	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
11	RM7076	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

Table 6. continued

12	RM3586	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
13	RM280	A	A	H	B	A	H	H	H	H	H	B	H	B	H	A	B	B	H	H	H	A	B	H	B	H	B	B	B
14	RM252	A	B	A	B	H	H	H	H	A	H	H	B	H	B	B	B	B	B	B	B	B	B	B	B	B	A	H	B
15	RM518	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
16	RM169	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
17	RM163	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
18	RM164	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
19	RM13	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
20	RM6836	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
21	RM225	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
22	RM7555	A	B	B	B	B	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
23	RM366	A	A	A	A	B	A	H	B	H	A	B	H	B	B	H	H	H	A	A	H	H	H	B	A	B	B	B	B
24	RM447	A	A	A	B	B	B	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
25	RM256	A	A	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
26	RM242	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
27	RM201	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B

Table 6. continued

28	RM6100	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
29	RM254	A	B	A	B	A	A	H	B	H	H	H	B	B	B	B	H	B	B	B	B	B	B	B	B	B	H	A	B
30	RM552	A	B	A	B	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B
31	RM26212	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
32	RM3701	A	H	B	A	A	A	H	A	H	H	A	H	H	H	H	H	A	H	H	B	H	B	H	A	A	A	H	B
33	RM224	A	B	B	B	B	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
34	RM19	A	B	B	B	H	H	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
35	RM17	A	H	B	B	A	A	B	B	A	A	B	A	B	A	H	H	H	H	H	B	A	B	A	A	B	B	B	B

A –Allele similar to parent N22 B – Allele similar to female parent Uma H – Heterozygous loci

4.5. Morphological screening of F_5 population for heat tolerance

Selfing acts as powerful way for achieving homozygosity at the gene loci in a heterozygous individual. To attain homozygosity in the heterozygous loci and stability in spike fertility percentage, 26 most fertile lines from F_4 were forwarded to raise F_5 plants. Hundred seeds from each of the selected twenty-six lines of F_5 having more than 75 % spike fertility percentage were kept for soaking. The 75 survived plants along with the parents Uma and N22 were evaluated for heat tolerance. No plants survived in two selected lines (16.6 and 31.1). Morphological observations were recorded to analyse spikelet fertility percentage and the resemblance of F_5 progenies to both the parents. Morphological characters studied among survived F_5 progenies of Uma x N22 are presented in Appendix II.

4.5.l. Plant height (cm)

The plant height ranged from 45 (Plant no.31.9.1) to 105 cm (Plant no. l6.1.4) with an average height of 74.77 cm. Uma had an average height of 82.3 cm while N22 had 105.6 cm height.

4.5.2. Tillers/plant

Total number of tillers per plant varied from 5 (Plant no.s 16.7.1, 31.6.8, 31.11.1, 31.12.2) to 14 (Plant no.15.3.2) with a mean of 9.36. Tillers per plant in both Uma and N22 was 12.

4.5.3. Panicles/plant

Number of panicles per plant ranged from 2 (Plant no.41.5.1 and 41.6.2) to 9 (Plant no.15.2.4, 15.2.5, 31.2.2) with a mean of 5 in F_5 plants. Uma had 8 panicles per plant while in N22 it was 10.

4.5.4. Panicle length (cm)

Panicle length ranged from 8.17 (Plant no.31.7.1) to 21.60 cm (Plant no.16.1.1) with an average of 14.52 cm in F_5 plants. Panicle length in Uma and N22 was 18.63 cm and 19.66 cm respectively.

4.5.5. Days for flowering

The days taken for appearance of first panicle ranged from 70 days (Plant no.16.5.2) to 87 days (Plant no.31.7.1) with an average of 77.96 days in F_5. First panicle in Uma was observed after 90 days while in N22 first panicle was observed 75 days after sowing.

4.5.6. Spikelets /panicle

The number of spikelets per panicle ranged from 27.33 (Plant no. 16.7.2) to 150.67 (Plant no.31.2.2) with an average of 78.07 in F_5 plants. The number of spikelets in Uma was 99.3 and 106.6 respectively.

4.5.7. Filled grains/panicle

The highest number of filled grains per panicle (74) was recorded in Plant no.31.2.2. Average number of filled grains/panicle was 26.91 in F_5 plants. mean filled grains per panicle in Uma was 84 while in in N22 it was 92.

4.5.8. Sterile grains/panicle

Sterile grains per panicle ranged from 15.67 (Plant no.31.9.1) to 117 (Plant no.15.2.2) with an average of 44.62 in F_5 plants. Average no. of sterile grains per panicle in Uma and N22 was 11.

4.5.9. 100 seed weight (g)

Average 100 seed weight was 1.65 g. The trait ranged between 0.5g (Plant no. 15.1.2) to 2.9 g (Plant no.31.2.5) in F_5 plants. Uma and N22 had a 100 seed weight of 2.9 g and 2.5 g respectively.

4.5.10. Grain weight /plant (g)

Average grain weight or yield per plant in the F_4 population was 3.99 g. grain weight per plant in Uma and N22 was 7.9 g and 7.8 g respectively.

4.5.11. Spikelet fertility %

Spike fertility percentage ranged from 5.38 % (Plant no. 15.1.2) to 70.78 % (Plant no.16.1.5) with a mean of 41.46 % in F_5 plants. Uma had 88.16 per cent while N22 had 89.26 per cent spikelet fertility.

4.6. Evaluation of selected F_5 progenies for morphological characters, yield parameters, trait similarities with Uma and N22

The expression of traits of economic importance is not only influenced by the genetic background but also to some extent by the external environment. Hence, resorting to both the genotypic and phenotypic selection, gives better output in breeding programmes.

Table 7. Phenotyping of F_5 plants with more than 60 % spikelet fertility

Trait	Maximum	Minimum	Mean
Plant height (cm)	105	63	82.66
Tillers/plant	12	7	9.60
Panicles/plant	8	3	5.20
Panicle length (cm)	21	12	15.88
Days for flowering	85	76	79.33
Spikelets /panicle	114	76	93.07
Filled grains/panicle	60	44	52.18
Sterile grains/panicle	44	23	33.66
100 seed weight (g)	2.9	1.9	2.13
Grain weight/plant (g)	7.1	4.1	5.48

The heat-tolerant accessions showing tolerance at booting and flowering stage are considered to be useful genotypes for the breeding program to improve heat resilience. Spikelet fertility is the major trait for identification and selection of plants for heat tolerance

F_5 plants showed 41.46 per cent mean spikelet fertility. Nine F_5 plants (Plant nos. 16.1.4, 16.1.5, 16.1.6, 31.2.3, 31.2.4, 31.2.5, 31.5.1, 31.6.6, and 31.6.9) with more than 60 per cent spikelet fertility were genotyped further using background markers for Uma. Since the F_5 plants and parents were evaluated in the rainy season, Uma (88.16 per cent) and N22 (89.26 per cent) had comparable spikelet fertility. The days taken for the appearance of first panicle in the selected F_5 lines (79 days) was similar to the early maturing parent N22. No. of spikelets per panicle and filled grains per panicle in the F_5 plants were lower compared to the parents grown along with them. Sterile grains per panicle was concordant in both the parents (11). Sterile grains per panicle in the selected F_5 plants (33.66) was higher compared to both the parents.

The F_5 lines also had some undesirable traits like relatively small-sized grains and seedlings were with weak and long stems as that of N22. Both parents and F_5 plants were highly susceptible to brown plant hopper. Ullala (Flonicamid 50 WG) at 3mg/l concentration was sprayed for controlling BPH. Grain discolouration due to fungal infection was also observed in spikelets of F_5 plants. Bavistin (1%) was sprayed against fungal infection. F_5 plants were severely affected with bacterial leaf blight during seedling stage. Pseudomonas at seedling stage and antibiotic Cyclin -50 at 0.6 mg/l was sprayed for controlling bacterial leaf blight in F_5 plants.

4.7. Genotyping of F_5 plants

4.7.l. Quantity and quality analysis of genomic DNA of parents and F_5 plants

Quantity and quality for the genomic DNA of F_5 plants and parents were analyzed. The A_{260}/A_{280} ratio of genomic DNA isolated from F_5 plants ranged from l.82 to 2.15, while the average value of parents Uma and N22 ranged from l.90 and l.92 respectively.

The quantity of the DNA isolated from the F_5 progenies varied from 249.5 µg/ml to l480.7 µg/ml. In the case of parents Uma and N22 the concentration of DNA extracted was 907.74.00 µg/ml and 86l.446 µg/ml respectively. The concentration of DNA (50 µg/ml DNA) required for genotyping was obtained by dilution of the samples

based on optical density (OD) values and DNA quantity (µg/ml) as per the procedure elaborated under section 3.4.4.

Table 8. Quality and quantity of genomic DNA of F_5 plants and parents

Genotype	Quantity of DNA (µg/ml)			Quality of DNA (A_{260}/A_{280})		
	Max	Min	Mean	Max	Min	Mean
N22 (Donor parent)	989.2	687.27	861.446	2.05	1.84	1.92
Uma	979.64	750.24	907.04	1.95	1.83	1.90
F_5 plants	1375.3	249.5	731.17	2.15	1.82	2.01

4.7.2. Foreground selection of F_5 population

All the 26 selected plants of F_4 registered only one amplicon (157bp) corresponding to the heat tolerant parent N22. Since F_5 progenies of these lines were forwarded and raised to get F_6 seeds, the presence of putative linked marker RM5749 was confirmed in all the nine selected F_5 progenies evaluated in the study. (Plate.7)

4.7.3. Allele distribution in the selected F_5 plants

The present study aimed to improve the agro-morphological character along with enhanced spikelet fertility and impart heat tolerance in the elite variety Uma (Mo 16).

A set of 35 Rice Microsatellite (RM) SSR markers distributed across the 12 chromosomes of rice were initially used to allelic distribution among the parents Uma and N22.

Of the 35 polymorphic SSR markers used, ten markers (RM13, RM163, RM164, RM166, RM169, RM212, RM302, RM518, and RM7076) exhibited the same allelic pattern of the female parent (Uma) in the 26 plants of F_4 having high spike fertility percentage indicating that the F_4 and the female parent had identical alleles at these marker loci.

Plate 7. Foreground selection using RM5749 in 9 selected plants of F_5

Monomorphism was observed in both the male parent (N22) and the 26 selected plants of F_4 studied in 9 (RM9, RM201, RM225, RM242, RM495, RM3586, RM6836, RM26212, and RM6100) out of 35 markers used for background selection indicating that the most fertile of F_4 and N22 had identical alleles at these marker loci.

Since the 26 selected lines of F_4 were selfed to raise F_5 plants, all its progenies segregate in the same allelic pattern for all homozygous loci as showed in F_4 generation. Thus, allelic patterns for these nineteen RM primers in the nine selected plants of F_5 were confirmed as same as that observed in F_4 generation.

The nine selected plants of F_5 with more than 60 percent spikelet fertility (Plant nos.16.1.4, l6.l.5, l6.l.6, 31.2.3, 31.2.4, 31.2.5, 31.5.l, 31.6.6, and 31.6.9) were genotyped with markers which showed heterozygous loci for the parents in F_4 generation. (Plate 8.1 to 8.4)

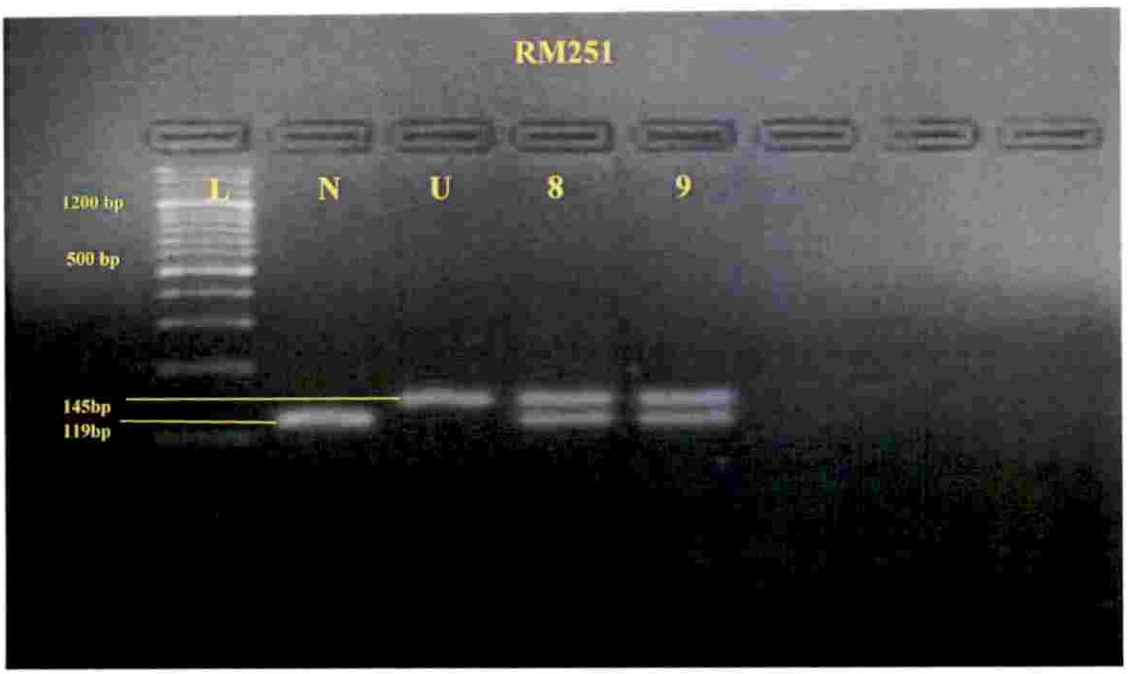
Background selection in the selected most fertile nine F_5 plants of Uma x N22 revealed that plant nos. 31.2.3 and 31.2.5 were similar to female parent Uma at 22 marker loci. Plant nos. 31.2.4, 31.5.1, 31.6.6, and 31.6.9 were similar to Uma at 21 marker loci. Also plant nos. l6.l.5, l6.l.6 showed similar to Uma at l8 marker loci. And plant no. l6.l.4 of F_5 were similar to Uma at 20 marker loci.

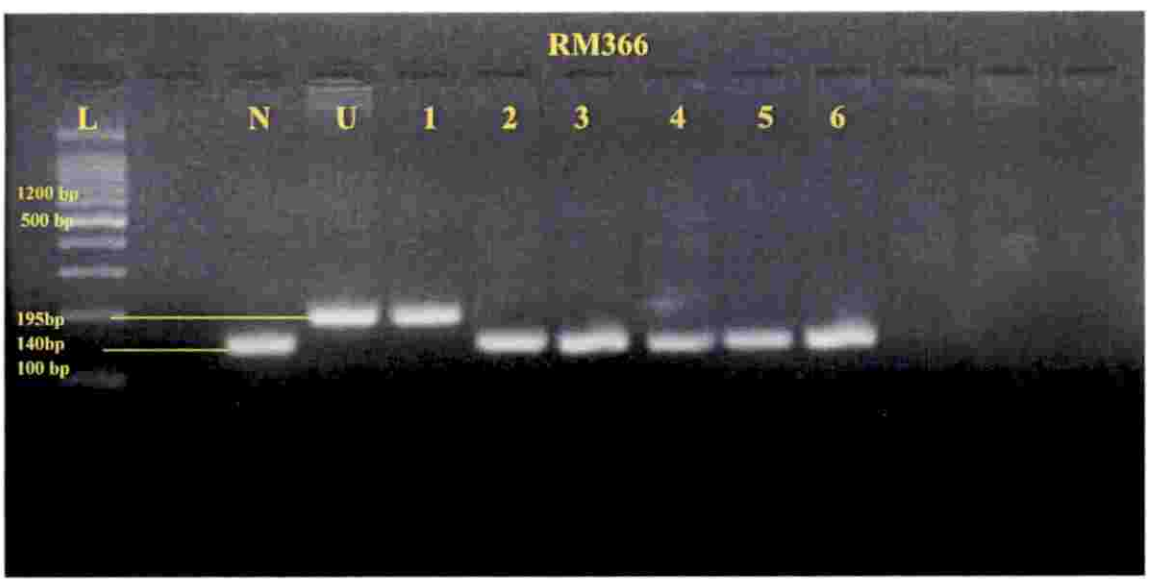
Table 9. Marker loci similar to parent Uma in selected F_5 plants

Plant nos.	No. of marker loci similar to Uma
31.2.3, 31.2.5	22
31.2.4, 31.5.l, 31.6.6, & 31.6.9	21
l6.1.5, l6.1.6	18
l6.1.4	20

Plant nos. 16.1.5, 31.6.9 were found to be heterozygous at 2 marker loci whereas 4 plants (plant nos. 16.1.6, 31.2.5, 31.5.1, 31.6.6) were heterozygous at a single marker locus (Table 9).

92

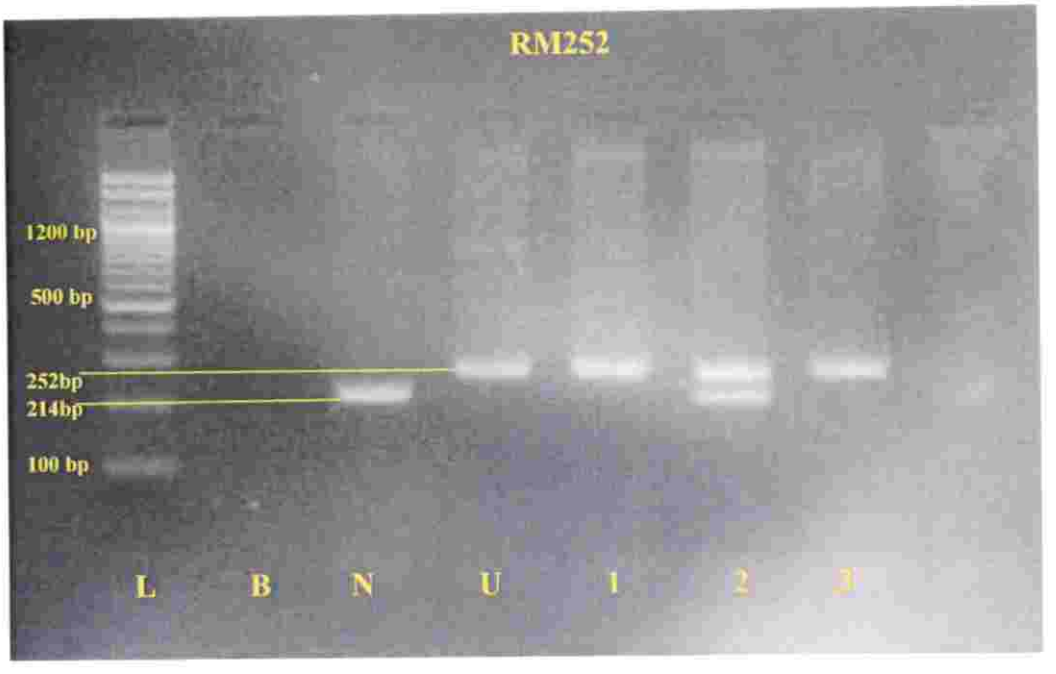


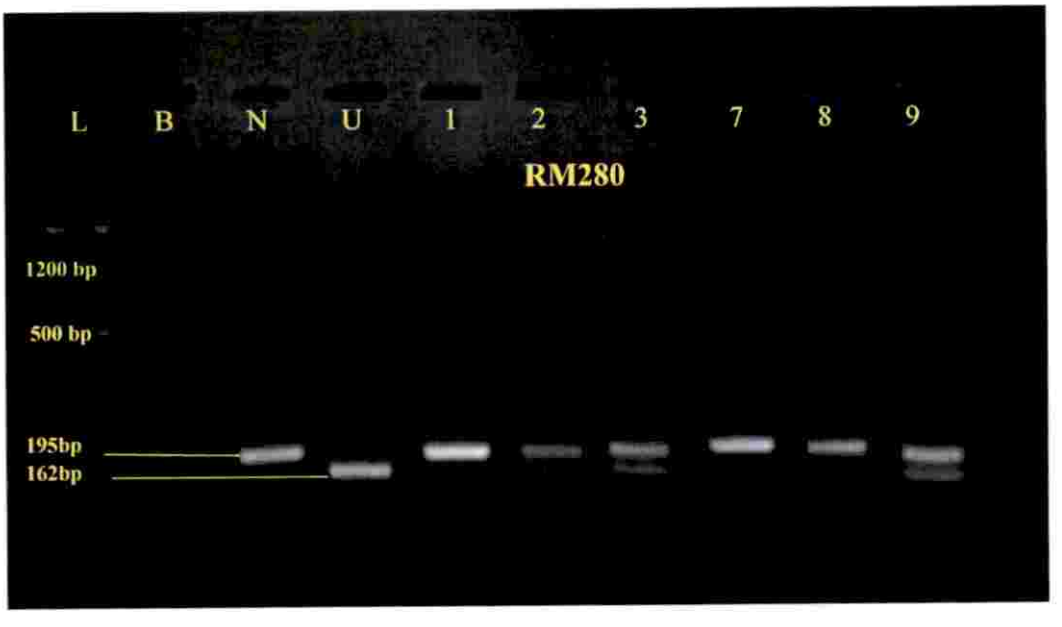


L: Ladder N: N22 U: Uma

1-16.1.4 2-16.1.5 3-16.1.6 4-31.2.3 5-31.2.4 6-31.2.5 8-31.6.6 9-31.6.9

Plate 8.1. Background selection of the selected plants in F_5 using RM251 and RM366

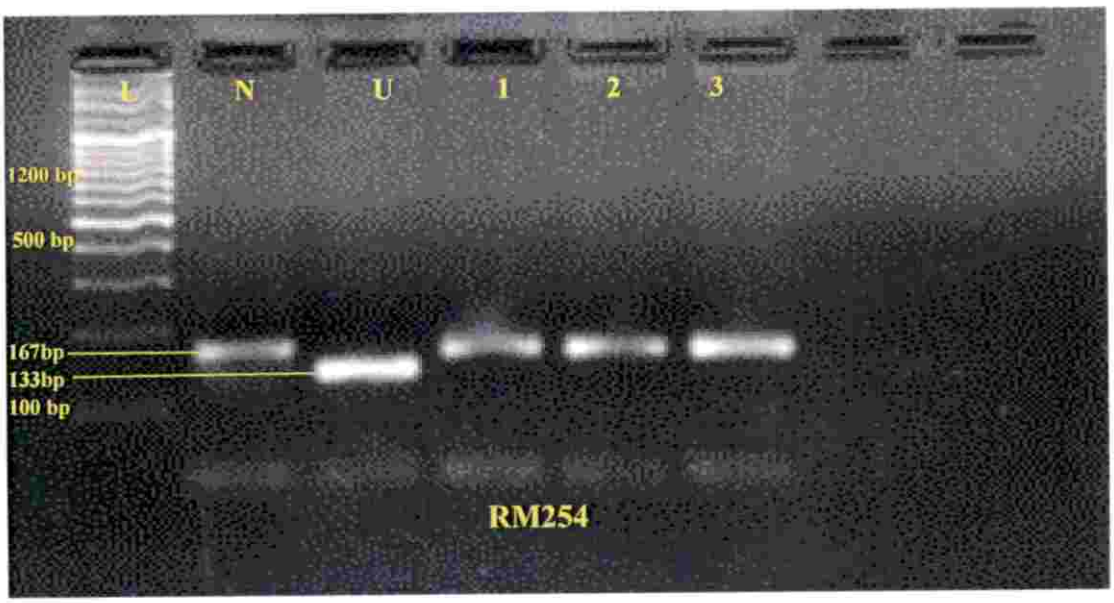


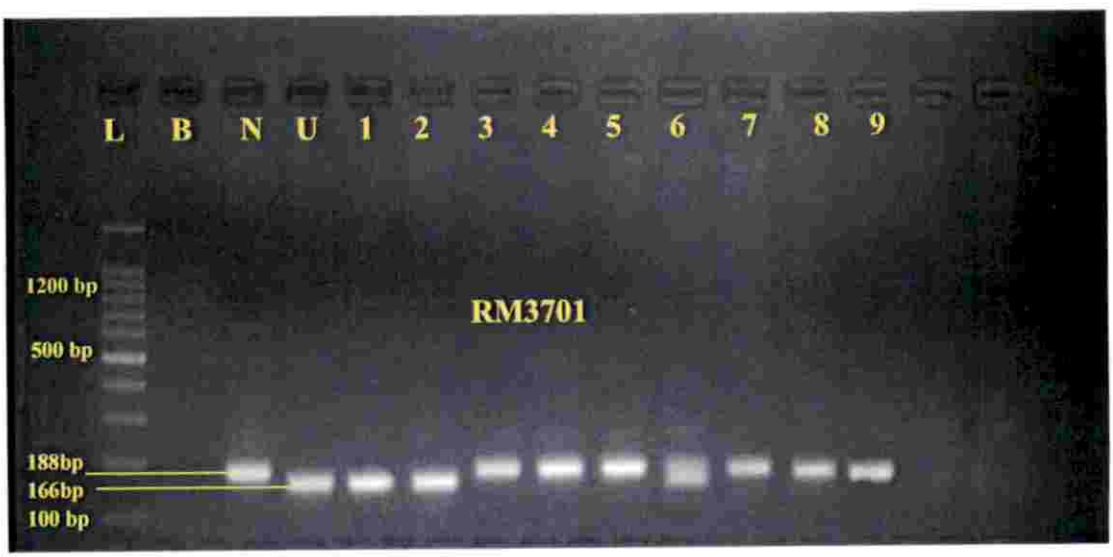


L: Ladder N: N22 U: Uma

1-16.1.4 2-16.1.5 3-16.1.6 7-31.5.1 8-31.6.6 9-31.6.9

Plate 8.2. Background selection of the selected plants in F_5 using RM252 and RM280





L: Ladder N: N22 U: Uma

1-16.1.4 2-16.1.5 3-16.1.6 4-31.2.3 5-31.2.4 6-31.2.5 7-31.5.1 8-31.6.6 9-31.6.9

Plate 8.3. Background selection of the selected plants in F_5 using RM254 and RM3701

L: Ladder N: N22 U: Uma

4-31.2.3 5-31.2.4 6-31.2.5 7-31.5.1 8-31.6.6 9-31.6.9

Plate 8.4. Background selection of the selected plants in F_5 using RM17

Plant no.16.1.6 was similar to N22 at 16 marker loci. Similarly, 15 marker loci were similar to N22 in plant no.s 16.1.4, and l6.1.5. Fourteen marker loci similar to N22 were found in plant no.31.2.4. Thirteen marker loci were identical to N22 were present in plant no.s 31.2.3, 31.5.1 and 31.6.6. The plant no.s 31.2.5 and 31.6.9 were identical to the donor parent N22 at 12 marker loci only.

Table 10. Marker loci similar to parent N22 in selected F_5 plants

Plant nos.	No. of marker loci similar to N22
16.1.6	16
16.1.4, 16.1.5	15
31.2.4	14
31.2.3, 31.6.6, 31.5.1	13
31.2.5, 31.6.9	12

The result thus indicated that among the most fertile 9 F_5 plants (Plate no.9) with RM5749 marker locus similar to N22, two plants 31.2.5 and 31.6.9 had a higher genome background recovery of female parent Uma.

Table 11. Distribution of alleles of marker loci used for background selection in the selected F_5 plants

Sl. No.	RM Marker	N22	l6.1.4	l6.1.5	l6.1.6	31.2.3	31.2.4	31.2.5	31.5.1	31.6.6	31.6.9	UMA
1	RM302	A	B	B	B	B	B	B	B	B	B	B
2	RM212	A	B	B	B	B	B	B	B	B	B	B
3	RM495	A	A	A	A	A	A	A	A	A	A	B
4	RM10346	A	B	B	B	B	B	B	B	B	B	B
5	RM9	A	A	A	A	A	A	A	A	A	A	B
6	RM3340	A	B	B	B	A	A	A	A	A	A	B
7	RM166	A	B	B	B	B	B	B	B	B	B	B
8	RM251	A	A	A	A	B	B	B	B	H	H	B
9	RM208	A	A	A	A	A	A	A	A	A	A	B
10	RM85	A	B	B	B	B	B	B	B	B	B	B

11	RM7076	A	B	B	B	B	B	B	B	B	B	B
12	RM3586	A	B	B	B	B	B	B	B	B	B	B
13	RM280	A	A	H	H	A	A	A	A	A	H	B
14	RM252	A	B	H	B	B	B	B	B	B	B	B

15	RM518	A	B	B	B	B	B	B	B	B	B	B
16	RM169	A	B	B	B	B	B	B	B	B	B	B
17	RM163	A	B	B	B	B	B	B	B	B	B	B
18	RM164	A	B	B	B	B	B	B	B	B	B	B
19	RM13	A	B	B	B	B	B	B	B	B	B	B
20	RM686	A	A	A	A	A	A	A	A	A	A	B
21	RM225	A	A	A	A	A	A	A	A	A	A	B
22	RM755	A	B	B	B	B	B	B	B	B	B	B
23	RM336	A	B	A	A	A	A	A	A	A	A	B
24	RM447	A	A	A	A	B	B	B	B	B	B	B
25	RM256	A	B	B	B	B	B	B	B	B	B	B
26	RM242	A	A	A	A	A	A	A	A	A	A	B
27	RM201	A	A	A	A	A	A	A	A	A	A	B
28	RM6100	A	A	A	A	A	A	A	A	A	A	B
29	RM254	A	A	A	A	B	B	B	B	B	B	B
30	RM552	A	A	A	A	B	B	B	B	B	B	B
31	RM26212	A	A	A	A	A	A	A	A	A	A	B
32	RM3701	A	B	B	A	A	A	H	A	B	B	B
33	RM224	A	A	A	A	B	B	B	B	B	B	B
34	RM19	A	B	B	B	B	B	B	B	B	B	B
35	RM17	A	B	B	B	B	A	B	H	B	B	B

A –Allele similar to parent N22 B – Allele similar to female parent Uma H – Heterozygous loci

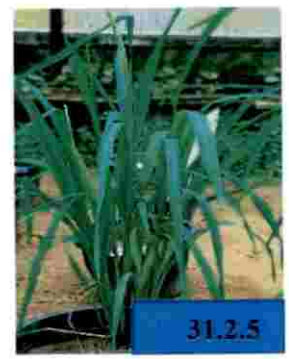


Plate 9. The nine selected plants from F_5 population of UMA x N22

4.7.4 Contribution of parental genome in the selected F_5 plants

The highest recovery of the parental genome of Uma was found in F_5 plant no. 31.2.5.(64.28%). Percentage contribution of parental genome of Uma in the selected plants of F_5 of Uma x N22 are presented in Table 10. The GGT software output indicated that maximum parent genomic regions of Uma among were present on chromosomes 3, 5 and 12. In parallel to the findings of the present study, Buu *et al.* (2014) reported that markers associated with heat tolerance were located mostly on chromosomes 3, 4, 6, 8, l0 and 11. Thus, the result of the Graphical genotyping software GGT version 2.0 also indicated a greater similarity between Plant No.3l.2.5 and parental genome of Uma (Figure 2)

Table 12. Percentage contribution from genome of parent Uma

Plant nos.	Percent contribution from genome of Uma
31.2.5	64.28
31.2.3 and 31.6.9	62.85
31.5.1 and 31.6.6	61.42
31.2.4	60.00
16.1.4	57.14
l6.1.5	54.28

In the present study heat tolerance was scored under natural heat stress in the field conditions based on IRRI spikelet fertility classification. F_4 lines with less than 25 percent spikelet sterility were forwarded to the raise F_5 plants of the cross Uma x N22.

Similar to the present study, Lang *et al.* (2015) used five *indica* varieties, OM5939, AS996, IR66, Gayabyeo (high yielding and susceptible to heat) and IKO547 (resistance to brown planthopper possessing *Bphl8*) were used as recurrent parents to develop heat tolerant lines. Two germplasm N22 and Dular were used as donor of QTL for heat tolerance. They used two markers RM3586 and RMl60 on

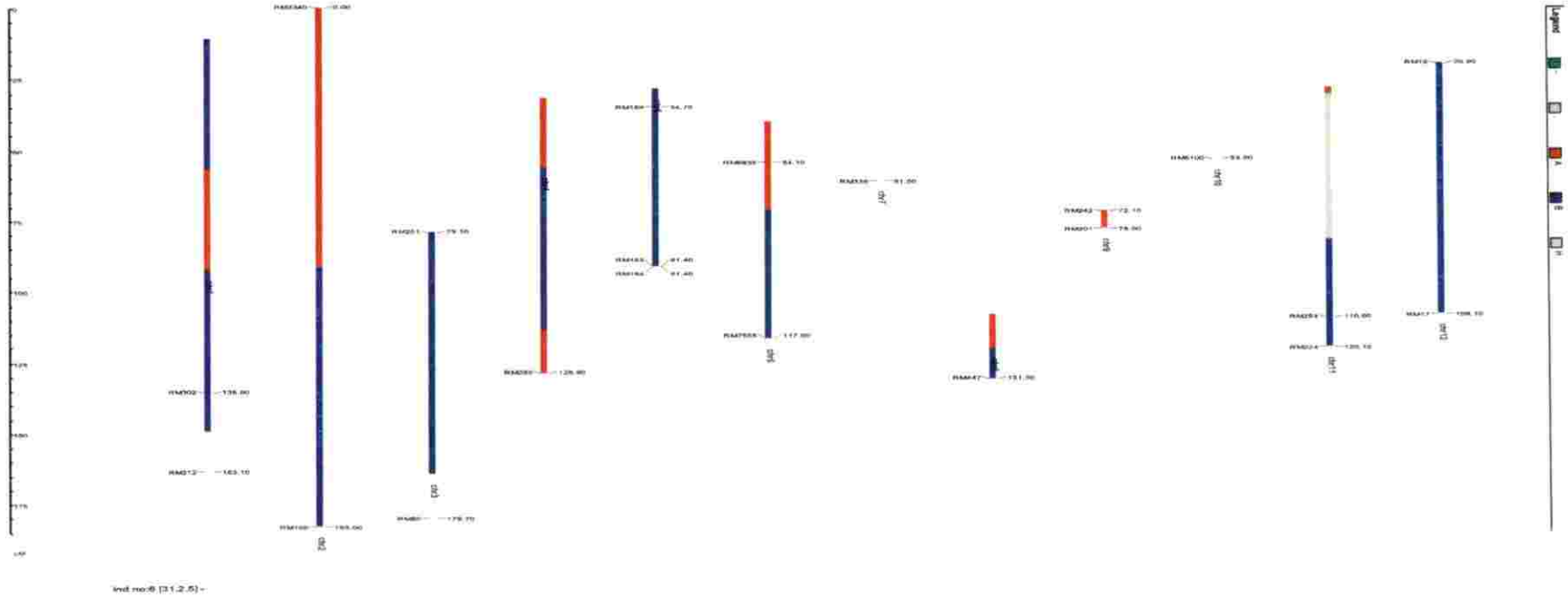


Figure 2. GGT software ouput for plant no. 31.2.5

chromosome 3 and four markers RM3735, RM3471, RM3687 and RM3536 on chromosome 4 to select promising lines in backcrossing populations for heat tolerance at flowering stage in rice. To evaluate heat tolerance at the reproductive period fifty lines selected in BC_3F_2, BC_4F_1, and BC_4F_2 and parents, plants were planted in field under natural heat stress and greenhouse. Heat tolerance scoring under field condition was based on percentage of unfilled grains. All the selected lines exhibited their homozygous alleles with two heat tolerant germplasm N22 or Dular in QTL. Twelve lines harboring homozygous alleles to QTL RM3586 on chromosome 3 and RM3735 on chromosome 4, respectively were selected and evaluated to agronomic traits and yield potential and finally four backcross lines were selected.

Since the parents Uma and N22 are susceptible brown plant hopper and bacterial leaf blight, both the F_4 and F_5 segregants of the cross Uma x N22 evaluated in the study were also highly susceptible to brown plant hopper and bacterial leaf blight. Bahuguna *et al.* (2015) identified that Nagina22 possessed undesirable traits like relatively small-sized grains and weak and long stems, a trait that leads to lodging of the plant and loss of grain yield. N22 also possessed desirable morphological and physiological characters like early maturity and high capacity for regeneration. Parallel to this finding, the plants evaluated in the present study had weak and long stem. The days for the appearance of first flower in the F_4 and F_5 plants of Uma x N22 were 77.42 and 79.33 days respectively which is indicative for the earliness which can be due to the influence of male parent N22 used in the crossing programme.

According to Manigbas *et al.* (2014) heat tolerant varieties such as N22 and Dular, usually have low yield potential and undesirable plant characteristics but by combining them with high yielding and improved rice varieties, new heat tolerant rice genotypes with high yield potential can be developed. Bo *et al.* (2012) studied heat resistance during flowering in 100 hybrid combinations. The rice plants were subjected to heat stress at 38 °C for 3 days as 50 % spikelets flowered. Analysis of origin of combinations with different heat tolerance indicated that most hybrids derived from the same female

parent showed similar heat resistance. It indicated female parent played a more important role in heat tolerance of hybrid rice.

From the findings of the above study, the subtle decrease in the mean spikelet fertility percentage in the F_4 (64.77 %) to F_5 (41.46 %) generation can be due to the influence of heat susceptible female parent Uma. Thus, higher heat tolerance scores can be expected from cross combinations with heat tolerant varieties as the female parent.

Thus, the nine selected lines with more than 60 per cent spikelet fertility selected in the present study are to be evaluated further through MAS as well as characterised agro-morphologically for phenotypic acceptability, as well as yield traits and yield to isolate novel high yielding genotypes with better heat tolerance.

Summary

V. SUMMARY

The study entitled 'Marker assisted selection for heat tolerance in rice (*Oryza sativa* L.)' was carried out in the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara during the period 2017-2019. Uma is among the most widely cultivated high yielding rice varieties in Kerala. However, yield loss due high temperature induced spikelet sterility is a recurring phenomenon during the third cropping season in Kole lands and rice growing tracts of Kuttanad. Seven lines of F_3 which were reported to be tolerant to heat stress served as the base material for the study. The objective of the study was to raise F_4 and F_5 generations of the cross Uma X N22 and to develop backcross lines. The research programme comprised of two experiments *viz.*, I) Raising of F_4 and F_5 population of Uma x N22 along with development of backcross lines using F_4 lines with Uma as the male parent, II) Marker assisted selection – foreground selection using RM5749 and background selection using the markers polymorphic between the parents. The salient findings of the study are summarized below.

1. Seven heat tolerant F_3 lines were raised to F_4
2. Fifty-nine survived F_4 plants and parents Uma and N22 which were grown in pots evaluated for morphological characters.
3. Grain filling was poor in F_4 plants and they were highly susceptible to BPH.
4. Twenty-six F_4 plants with more than 75 % spikelet fertility were selected. Among the 26 plants four plants were having more than 90 % spikelet fertility percentage.
5. Marker assisted selection was carried out using RM5749 for foreground selection and 35 markers polymorphic between the parents were used for background screening

6. Twenty-four F_4 families were forwarded to F_5 generation, two lines didn't survive.
7. Seventy-five survived F_5 plants were evaluated for spikelet fertility percentage and other morphological characters
8. All the 9 selected plants registered only an amplicon corresponding to the heat tolerant parent N22.
9. Background screening carried out with polymorphic markers
10. All the nine F_5 plants showed 60-70 % spikelet fertility and 54-64 % similarity to Uma genome

As a future line of work, MAS coupled with phenotypic characterization of advanced generation of selected plants in this study will help to isolate genotypes with heat tolerance and resembling Uma. But, other agronomic traits like grain filling in spikelets need to be considered while selection of advanced generations Thus, the segregants with heat tolerance can be used in future breeding programmes.

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Appendices

Appendix - I

Plant no.	Plant height (cm)	Tillers /plant	Panicles /plant	Panicle length (cm)	Days for flowering	Spikelets / panicle	Filled grains / panicle	Partially filled grains / panicle	Sterile grains /panicle	100 seed weight	Grain weight / plant	Spikelet fertility
12.1	75	10	8	19.5	70	121.1	12.2	32.6	76.3	1.5	3.1	37
12.2	69.5	14	5	16	69	111.8	23.8	35.4	52.6	2.5	4.3	52.96
12.3	78	10	8	18.5	70	89.2	15.2	36.4	37.6	1.9	3.8	57.85
12.4	80	11	11	19	75	128.2	31	50.2	47	2	4.6	63.34
13.1	93.5	8	10	20.5	69	149.4	32.4	54.6	62.4	2.3	5.9	58.24
13.2	90	12	7	19.2	68	121.7	25.8	35.4	60.5	2.7	5.3	50.29
13.3	85	10	8	17	69	109	17.4	58.2	33.4	1.9	4.8	69.36
15.1	81	11	10	22.5	85	138	97.3	12.4	28.3	1.94	7.3	79.5
15.2	78.2	10	8	19.6	82	111.2	90.6	8.2	12.4	1.99	7.10	88.85
15.3	80	8	9	20.5	80	164.8	108.6	27.8	28.4	1.85	7.6	82.77
15.4	82.5	9	10	21.2	81	109	83.2	10.4	15.4	2.01	7.08	85.88
15.5	76	10	8	21	81	129.3	75.4	21.2	32.7	1.58	4.01	74.71
15.6	79.4	9	9	19.8	80	69.2	30	13.8	25.4	2.12	5.8	63.3
15.7	79	9	8	22	80	97.8	53.6	26	18.2	1.73	6.6	81.4
15.8	80.5	10	10	21.4	79	37.8	12	4	21.8	1.91	6.9	42.33
16.1	81.3	7	7	20.2	69	127	105.4	12	9.6	1.98	8.21	92.45
16.2	89	8	8	19.1	73	118.7	84	20.5	14.2	1.94	7.3	88.04
16.3	83.5	9	11	20.5	75	129.6	100	6	23.6	2.11	7.50	81.8
16.4	80.1	8	10	20.2	70	117.6	73.2	29.4	15	1.68	6.51	87.25
16.5	82	8	9	22	73	117.5	62.8	38	17.5	1.45	4.53	85.11
16.6	79.5	7	8	18.6	75	130.6	82	16.4	32.2	1.9	3.16	75.35
16.7	76	9	9	19	76	118.4	78	26.4	14	2.7	4.64	88.18
16.8	80	8	6	22	76	175.2	80	22	73.2	2.22	5.93	58.22
16.9	81.5	7	8	19.5	75	179.3	88.3	21	70	1.67	4.5	60.96
16.1	83.2	8	8	18.8	75	147.5	85.4	23	39.1	1.75	4.11	73.5
31.1	80.2	8	7	19.5	81	105.3	48.6	34.2	22.5	2.04	6.1	78.64
31.2	87	10	8	19.6	77	136.6	108	18.4	10.2	2.35	7.91	92.54
31.3	73.6	8	11	20	78	149.4	104.4	31.2	13.8	1.97	5.7	90.77
31.4	82	9	10	23	77	139	91	32.6	15.4	1.9	6.81	88.93

Plant no.	Plant height (cm)	Tillers /plant	Panicles /plant	Panicle length (cm)	Days for flowering	Spikelets / panicle	Filled grains / panicle	Partially filled grains / panicle	Sterile grains /panicle	100 seed weight	Grain weight / plant	Spikelet fertility
31.5	79.5	8	9	19.5	75	132.8	80.2	41.6	11	1.95	6.54	91.72
31.6	79	10	9	19.2	79	174.4	130.2	8	36.2	1.94	8.10	79.25
31.7	80.2	9	8	19.7	77	42.2	28	5.6	8.6	2.12	5.8	79.63
31.8	86.5	9	7	22	80	106.8	63.2	26	17.6	1.7	4.57	83.53
31.9	76.4	8	7	23.5	75	124.8	79.4	15.4	30	1.3	5.91	75.97
31.1	77	7	9	19.5	79	114.6	59	34.8	20.8	2.6	4.98	81.85
31.11	80	8	8	19.8	77	93.9	61.5	13	19.4	2.4	6.53	79.34
31.12	74.5	12	10	17	83	128.8	90.6	23.8	14.4	1.8	7.3	88.82
41.1	90	7	8	20.8	81	141	0	28	113	0	0	19.86
41.2	86.2	8	9	19.2	81	161	16.6	8.2	136.2	1.79	3.65	15.41
41.3	90.3	7	6	22	78	166.2	0	0	166.2	0	0	0
41.4	84.5	8	8	20.9	81	192.4	79.2	31.8	81.4	1.84	4.9	57.7
41.5	99	8	7	23.7	80	133.8	75.8	34.5	23.5	1.64	4.7	82.44
41.6	82	9	8	22.4	76	149.6	18.6	110.6	20.4	1.32	3.95	86.37
41.7	78	8	7	18.6	76	121.8	12.6	32.8	76.4	2.41	4.55	37.28
41.8	82.5	8	8	22.4	83	109.1	17.5	58	33.6	1.84	4.29	69.21
41.9	79.6	9	8	17	85	92.9	15	47.2	30.7	1.36	4.07	66.96
41.1	80	7	8	20.6	80	146.1	28	71.6	46.5	2.75	5.01	68.18
41.11	85.2	8	8	22.6	79	165.6	45	30.2	90.4	3.71	5.4	45.42
41.12	79.5	10	7	18.8	79	149.3	32.4	54	62.9	1.96	4.28	57.81
45.1	87.5	8	8	19.6	77	139.4	23.2	5.4	110.8	1.8	3	20.52
45.2	82	8	7	19.8	79	144.3	15.6	8.5	120.2	1.1	3.1	16.71
45.3	85.5	9	8	20.5	77	231	99.2	20.4	111.4	1.7	3.8	51.78
45.4	86.2	9	8	17.9	76	102	8	13.1	80.9	0.8	3.5	20.69
45.5	82.2	8	7	22.2	76	138	9	36.4	92.6	0.5	1.2	32.9
45.6	90	11	8	18.5	79	72	14.8	21	36.2	2.2	3.9	49.73
45.7	77	9	8	16	80	90.6	44.2	16.4	30	1.1	4.2	66.89
45.8	82.6	10	8	20.5	80	192.4	79.2	31.8	81.4	1.9	5.8	57.7
45.9	82	9	8	18.4	79	83.6	40.2	15	28.4	1.4	3.6	66.03

Appendix - II

Plant no.	Plant height (cm)	Tillers /plant	Panicles /plant	Panicle length (cm)	Days for flowering	Spikelets / panicle	Filled grains / panicle	Partially filled grains / panicle	Sterile grains /panicle	100 seed weight	Grain weight / plant	Spikelet fertility
15.1.1	75	9	5	16.33	75	81.67	11.00	3.00	67.67	1.7	3.4	1.7
15.1.2	79	10	7	11.93	80	31.00	1.67	0.00	29.33	0.5	2.1	0.5
15.1.3	84	8	6	17.33	75	86.00	19.67	5.33	61.00	0.9	2.4	0.9
15.2.1	63	7	4	13.50	77	56.67	10.33	2.33	44.00	0.7	1.6	0.7
15.2.2	80	9	7	20.83	80	148.33	18.67	12.67	117.00	1.1	3.9	1.1
15.2.3	74	7	5	19.27	75	101.33	22.33	4.33	74.67	1.5	3.1	1.5
15.2.4	79	10	9	21.47	75	145.67	38.67	12.67	94.33	2.5	4.3	2.5
15.2.5	82	12	9	21.50	77	96.67	24.33	5.67	66.67	1.9	3.8	1.9
15.2.6	85	11	8	16.90	78	84.67	13.33	7.33	64.00	2	4.6	2
15.2.7	66	9	4	12.37	73	68.67	8.00	3.67	57.00	2.3	5.9	2.3
15.2.8	76	10	6	13.93	75	92.67	38.00	9.33	45.33	2.4	5.3	2.4
15.2.9	75	12	8	15.43	72	140.33	52.00	19.33	69.00	1.9	3.9	1.9
15.3.1	80	10	4	11.67	80	76.33	20.67	5.67	50.00	1.4	4.5	1.4
15.3.2	65	14	3	10.67	82	43.67	8.67	5.33	29.67	1.6	4.7	1.6
15.4.1	76	13	6	14.60	74	60.33	16.00	7.00	37.33	1.9	4	1.9
15.7.1	73	12	5	12.57	79	67.67	20.67	5.00	42.00	1.6	3.9	1.6
16.1.1	90	11	6	21.60	80	131.67	65.00	8.00	58.67	1.8	3.4	1.8
16.1.2	86	9	3	14.03	74	54.67	19.33	2.00	33.33	1.3	2.9	1.3
16.1.3	84	10	6	19.67	81	126.33	46.67	16.33	63.33	1.4	3.1	1.4
16.1.4	105	12	8	21.17	82	94.33	60.00	2.00	32.33	1.9	4.1	1.9
16.1.5	82	7	5	14.37	76	81.00	52.33	5.00	23.67	2.1	5.8	2.1
16.1.6	89	10	6	20.73	78	79.00	46.00	4.00	29.00	2.2	6.1	2.2
16.2.1	80	12	5	19.93	82	103.33	55.67	3.00	44.67	1.8	4.6	1.8
16.2.2	79	10	8	16.30	76	90.33	37.33	7.00	46.00	1.1	3.7	1.1
16.2.3	82	9	7	17.07	81	99.00	35.33	4.00	59.67	1.8	3	1.8
16.2.4	90	10	4	18.40	73	128.33	43.00	13.00	72.33	1.1	3.1	1.1
16.3.1	92	9	5	19.37	79	117.67	24.00	6.00	87.67	1.7	3.8	1.7
16.4.1	78	9	3	14.57	81	70.33	13.00	8.67	48.67	0.8	3.5	0.8
16.4.2	83	10	5	16.50	83	83.33	15.33	7.33	60.67	0.9	2.4	0.9

Appendix - II

Plant no.	Plant height (cm)	Tillers /plant	Panicles /plant	Panicle length (cm)	Days for flowering	Spikelets / panicle	Filled grains / panicle	Partially filled grains / panicle	Sterile grains /panicle	100 seed weight	Grain weight / plant	Spikelet fertility
16.5.1	76	6	3	15.30	77	93.67	13.00	5.00	75.67	1.9	3.9	1.9
16.5.2	73	9	7	17.33	82	74.67	13.33	6.67	54.67	1.1	4.2	1.1
16.7.1	75	5	3	17.80	70	79.67	20.67	6.00	53.00	1.9	5.8	1.9
16.7.2	79	6	3	9.00	73	27.33	6.67	3.00	17.67	1.4	3.6	1.4
31.2.1	99	12	8	19.20	79	98.33	45.33	6.00	47.00	0.9	2.9	0.9
31.2.2	90	11	9	20.47	75	150.67	74.00	11.00	65.67	1.1	3.8	1.1
31.2.3	85	10	7	16.67	79	105.00	55.33	10.00	39.67	2.1	5.1	2.1
31.2.4	80	11	5	14.37	76	76.00	45.00	5.33	25.67	1.9	6.8	1.9
31.2.5	85	10	5	12.60	79	82.67	44.00	10.00	28.67	2.9	7.1	2.9
31.3.1	88	12	6	12.60	75	65.67	24.00	9.00	32.67	1	2.9	1
31.3.2	79	9	4	12.27	81	53.33	16.00	8.33	29.00	1.1	2.8	1.1
31.3.3	75	12	6	13.43	80	58.00	24.67	5.67	27.67	1.04	2.1	1.04
31.4.1	68	9	3	10.07	79	45.33	19.33	5.00	21.00	1.5	3.1	1.5
31.4.2	70	10	4	11.40	79	52.33	18.00	5.33	29.00	2.4	4.3	2.4
31.4.3	62	11	6	14.80	76	68.00	24.67	7.67	35.67	1.9	3.8	1.9
31.5.1	89	9	4	16.37	80	114.00	59.67	9.67	44.67	2	4.6	2
31.5.2	85	10	4	16.50	81	97.33	41.67	4.67	51.00	2.3	5.9	2.3
31.5.3	72	9	8	15.57	73	80.33	34.33	4.33	41.67	1.9	3.2	1.9
31.5.4	78	11	9	15.30	75	94.00	44.33	11.00	38.67	2.4	4.6	2.4
31.5.5	90	8	7	13.70	80	94.67	39.00	9.33	46.33	2.2	5.9	2.2
31.6.1	92	8	5	14.03	75	97.67	36.33	10.33	51.00	1.1	3.5	1.1
31.6.2	86	9	4	15.03	82	87.33	30.33	7.33	49.67	0.9	2.2	0.9
31.6.3	84	6	5	13.70	80	103.33	24.67	12.00	66.67	0.9	2.7	0.9
31.6.4	89	9	6	12.00	80	63.00	27.00	7.67	28.33	1.2	2.1	1.2
31.6.5	81	10	6	9.37	81	32.67	11.00	3.00	18.67	1.1	4.2	1.1
31.6.6	66	9	3	12.53	79	98.33	51.67	8.00	38.67	2.1	5.2	2.1
31.6.7	79	8	4	11.33	82	90.33	36.33	12.33	41.67	1.2	3.4	1.2
31.6.8	70	5	3	14.10	76	93.33	25.67	7.33	60.33	1.1	3.5	1.1
31.6.9	63	9	4	14.13	85	107.33	55.67	11.00	40.67	2	4.6	2

Appendix - II

Plant no.	Plant height (cm)	Tillers /plant	Panicles /plant	Panicle length (cm)	Days for flowering	Spikelets / panicle	Filled grains / panicle	Partially filled grains / panicle	Sterile grains /panicle	100 seed weight	Grain weight / plant	Spikelet fertility
31.7.1	56	9	5	8.17	87	53.00	11.67	8.00	33.33	1	2.9	1
31.8.1	76	6	3	14.47	81	72.33	24.67	7.67	40.00	1.1	2.8	1.1
31.8.2	70	7	3	11.37	79	61.33	18.00	9.33	34.00	1.04	2.1	1.04
31.9.1	45	6	3	11.37	82	28.33	9.00	3.67	15.67	1.5	3.1	1.5
31.10.1	58	9	3	11.53	86	43.33	12.33	4.00	27.00	2.4	4.3	2.4
31.11.1	66	5	4	9.67	74	33.00	10.00	3.00	20.00	1.9	3.8	1.9
31.11.2	63	9	4	8.87	79	32.67	8.33	3.67	20.67	2	4.6	2
31.11.3	69	10	4	8.40	75	32.67	10.00	4.00	18.67	2.3	5.9	2.3
31.11.4	69	10	3	9.87	75	37.00	10.33	4.00	22.67	2.1	5.3	2.1
31.11.5	82	11	4	11.87	73	36.67	11.00	2.33	23.33	1.9	4.8	1.9
31.11.6	70	10	4	10.47	79	31.67	8.33	2.00	21.33	1.9	4	1.9
31.12.1	79	8	4	16.03	80	79.33	16.00	8.00	55.33	1.5	4.5	1.5
31.12.2	80	5	3	14.67	76	83.00	25.33	6.00	51.67	1.9	3.2	1.9

MARKER ASSISTED SELECTION FOR HEAT TOLERANCE IN RICE (*Oryza sativa* L.)

By,

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(2017-11-021)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

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Faculty of Agriculture

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2019

Abstract

Rice is highly susceptible to heat stress, particularly during the reproductive and ripening stages. In the major rice growing tracts of Kerala *viz*., at Palakkad, Kole and Kuttanad, the temperature tends to rise up to 40 °C or more during the second/third crop. Consequently, high temperature induced sterility has become a serious problem. To tackle this, high yielding varieties coupled with heat stress need to be developed. As most of the prevalent high yielding rice varieties in Kerala including Uma are highly susceptible to heat stress. It is therefore, essential to impart heat tolerance to such varieties which are cultivated to a very large extent.

Marker assisted selection (MAS) has been identified as a dependable, reproducible and time saving strategy to confirm the presence of desirable gene and to quicken the breeding cycle. A study conducted for the identification of SSR markers linked to the genes for heat tolerance in rice through bulked segregant analysis approach using F_3 population of the cross Uma x N22 revealed that microsatellite marker, RM5749 was tightly linked to spikelet fertility trait under heat stress.

The F_4 population (59 nos.) raised from seven F_3 lines that were found tolerant to heat stress comprised the base population for the present study. They were characterised morphologically and heat tolerance was scored under natural heat stress in the field conditions based on IRRI spikelet fertility classification. In the 26 F_4 lines that registered more than 75 per cent spikelet fertility, foreground selection was done using RM5749. All the 26 F_4 plants registered an amplicon corresponding to the heat tolerant parent N22. Background selection of these 26 lines was done using 35 markers found polymorphic between the parents Uma and N22.

Seventy five F_5 plants were evaluated for morphological characters. Among these, nine F_5 plants (Plant nos.16.1.4, 16.1.5, 16.1.6, 31.2.3, 31.2.4, 31.2.5, 31.5.1,

31.6.6, and 31.6.9) with high spikelet fertility (60-70 %) were selected and genotyped using RM5749. These lines were further genotyped using the 35 polymorphic background markers. All the nine F_5 plants recorded 54-64 % similarity to Uma genome. The highest spikelet fertility percentage was observed in plant no.16.1.5 (70.78 %) while the highest recovery of the parental genome of Uma was found in plant no. 31.2.5 (64.28 %).

Backcrossing of the seven selected heat tolerant lines of F_4 (lines 12, 13, 15, 16, 31, 41, and 45) with Uma as male parent resulted in production of BC_1F_1 seeds. However, the seedlings raised from these seeds did not survive under field conditions.

The results obtained thus indicated that the nine lines selected in the present study are to be evaluated in further generations morphologically inorder to isolate genotypes with tolerance to heat stress.