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Research Note

Validation of Newly Designed SSR Markers for Eight Rice (*Oryza sativa* L.) Genotypes with Variable Heat Tolerance Responses Based on Agromorphic Data and Pollen Fertility Analysis

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Rice is among the most valuable staple food crops in the world. However, several challenges greatly affect production, one of which is the threat imposed by heat stress. To address this, researchers are developing varieties that are tolerant to heat stress with the aid of genetic markers. In this study, eight rice genotypes, namely Dular, Nagina 22, NSIC Rc 222, Milyang 23, EL15, EL92, EL85, and IR52 were observed for agromorphic data, which included plant height, panicle length, filled and unfilled grains, and grain yield. Flower samples were collected to determine the effect of heat stress on pollen fertility. Molecular markers were designed via *in silico* analysis based on the nine QTL regions distributed among five chromosomes (1, 3, 4, 5, and 10). Out of the 90 newly developed markers, Markers 3066 and 2503 showed good potential as informative markers among heat tolerance classification. Results showed genotype-specific responses of varieties during heat stress and non-heat stress conditions. EL15 had the best agromorphic performance and appeared to be the best elite line. Identification of gene-specific SSR markers has proven to be effective in understanding heat tolerance for future marker-assisted selection.

Keywords: high-temperature, marker-assisted selection (MAS), microsatellites, *in silico* analysis, rice

Abbreviation: EL—elite lines, GMATA—Genome-wide Microsatellite Analyzing Toward Application, HT—heat-tolerant, IGV—Integrative Genomics Viewer, MINCER—Micrometeorological Instrument for Near Canopy Environment of Rice, NCT—National Cooperative Testing

INTRODUCTION

Climate change is caused by increasing the amounts of greenhouse gases that serve as a blanket to the earth's atmosphere, resulting in global warming (Jain 1993). However, warming does not refer to an average but to an excessive increase in temperature that creates long-lasting changes in all components of the climate system (Jain 1993). Despite the increasing number of climate change mitigation strategies, greenhouse gases have continued to accumulate from 1970 - 2010, with more massive absolute increases between 2000 and 2010 (IPCC 2014). Additionally, it was projected that heat waves and extreme temperatures would become more intense, frequent, and prolonged than in recent years (Meehl et al. 2007), which would adversely affect rice production. As the most consumed and produced staple, rice provides approximately 19% of the daily caloric supply (545 kcal) of the world's population (IRRI 2011). Rice consumption, therefore, has a direct effect on people's health and decreasing yields due to high temperatures can have enormous consequences.

The optimum temperature range for the healthy development of rice is 27 – 32°C (Peng et al. 2004; Prasad et al. 2006; Jagadish et al. 2014; Manigbas et al. 2014). Temperatures that are higher than the optimum induce

floret sterility thus decrease rice yield (Nakagawa et al. 2003). Above-optimum temperatures also affect almost all growth stages in rice (Shah et al. 2011). Reproductive stages such as anthesis and fertilization are the most sensitive while the booting stage has a lesser extent of susceptibility (Satake and Yoshida 1978; Farrell et al. 2006). Probable causes are the inhibition of pollen grain enlargement, indehiscence of anthers, and inadequate release of pollen grains (Matsui et al. 2000; Matsui 2005) resulting in fewer pollen grains intercepted by the stigma, thereby affecting fertilization and, eventually, yield. At the microspore stage, a minimum of 2 d of exposure to heat stress is enough to induce spikelet sterility and irreversibly deter panicle development (Endo et al. 2009). Specifically, high temperatures are mainly attributed to the inhibition of anther dehiscence, pollen sterility, and failed germination (Jagadish et al. 2010; Tenorio et al. 2013). However, studies on the effects of high temperatures and heat stress on vegetative development remain few compared to those on the reproductive stage (Prasanth et al. 2012).

To address this deficiency, breeders and researchers are developing varieties that are tolerant to certain types of stresses using genetic markers associated with heat tolerance. For instance, Marker-Assisted Selection (MAS) has been used to improve tolerance to stresses such as heat, drought, salinity, insects, and diseases using simple sequence repeats (SSR). SSR markers have a huge efficiency potential and are preferable for many forms of high throughput mapping, genetic analysis, and marker-assisted plant improvement strategies (McCouch et al. 1997; Cregan et al. 1999; Coburn et al. 2002). These markers are codominant, multi-allelic, and can be reliably utilized to analyze both germplasms of *Indica* and *Japonica* (Harrington 2000; Chen et al. 2002). Uses of SSR markers in plant breeding programs include the facilitation of the appropriate choice of parents for hybridization, the mapping of genes associated with economically important traits, and DNA fingerprinting (Gupta and Varshney 2000).

This study was conducted to investigate heat-tolerant varieties and three elite rice lines, and observe their morphometrics, which include plant height, number of productive tillers, panicle length, spikelet fertility, pollen fertility, and grain weight. In addition, newly designed molecular markers from GMATA were validated, and elite rice lines with the highest potential for the varietal improvement and broadening of the genetic base were recommended.

MATERIALS AND METHODS

Plant Materials

Based on the agromorphic traits such as yield performance, grain quality, and potential heat-tolerant traits (Selote and Khanna-Chopra 2004; Liu et al. 2006; Prasad et al. 2006; Jagadish et al. 2008; Jagadish et al. 2010), eight genotypes were used for the study: IR 52 (intolerant check), Nagina 22 or N22 and Dular (tolerant checks), Milyang 23 (moderately tolerant), and NSIC Rc 222 (yield check). Three NCT top elite lines, namely EL-85 (PR42130-M-1-B-6-2-B-7), EL-15 (PR 40330-4-2-7-1-2-1), and EL-92 (PR42132-M(I)-1-B-8-B-B-6), were also examined. The elite lines are stable lines from F_7 generations and have heat tolerance backgrounds. The varieties' characteristics are shown in Table 1.

Pot experiments were conducted from July to November 2019 at the Philippine Rice Research Institute (PhilRice) in Maligaya, Science City of Muñoz, Nueva Ecija. The seeds were sown in seed beds and were transplanted to plastic pots (L x W x H = 40 x 30 x 12 cm) filled with natural clay loam soil after 15 d. A total of 10 pots per variety per treatment with three replicates were grown in the glasshouse and in the growth chamber (Fig. 1). These varieties and elite lines are short-day and non-photoperiod-sensitive. Under glasshouse conditions with typically higher temperatures than field conditions, the temperature (°C), relative humidity (RH), and dew point (°C) were automatically recorded using Micrometeorological Instrument for Near Canopy Environment of Rice (MINCER) (Fukuoka et al. 2012) set to gather data every 2 min for 24 h everyday. The recommended fertilizer rate of 90N-60P_2O_4-60K_2O was applied in pots with 1 g per pot of complete fertilizer and

Table 1. Maturity and yield characteristics of the eight rice genotypes used in the study.

Varieties	Classification	Maturity (days)	Yield (th^{-1})	Yield Potential (th^1)
Dular	Heat-tolerant (V)	95	6.0	8.0
IR52	Heat-intolerant (V)	120	5.0	6.2
Milyang 23	Moderately tolerant (V)	120	7.5	7.4
Nagina 22 (N22)	Heat-tolerant (V)	95	2.0	5.0
NSIC Rc 222	Unclassified (V)	120	6.0	8.9
EL92	Unclassified (EL)	118	5.5	6.9
EL15	Unclassified (EL)	122	5.8	8.2
EL85	Unclassified (EL)	116	6.4	7.6

Source: Philippine Rice Research Institute, NCT trials. Yield values are under NCT trials. V -variety, EL- Elite line.

Fig. 1. Growth chamber setup used for the study. Environmental system Daihan Labtech Co., LTD. The defined environment conditions were maintained throughout the three trials.

0.08 g per pot of urea with standing water throughout the growth period. In the growth chamber experiment, at 15 d after sowing, plants were exposed to high temperatures for 3 d with 38°C ± 3 and 25°C ± 3 day and night temperatures, respectively, and 70 ± 5% relative humidity.

Four young leaves (approximately 4 cm long) from each treatment with three replicates each tagged plant were collected 5 d after exposure. Leaf samples were stored at −20 °C for DNA analysis.

Data Collection

Temperature and Relative Humidity

Air temperature and relative humidity data were monitored and collected using MINCER (Fukuoka et al. 2012) to check the temperature changes in the glasshouse setup. This device is a standalone force-ventilated system that uses a solar cell-powered ventilator with rechargeable batteries and a power control circuit (Fig. 2). A data logger placed inside the ventilated radiation shield PVC pipe detected and recorded the temperature (°C), relative humidity (%), and dew point (%) every 2 min. MINCER's air intake was set at the height of panicle.

Agromorphic Data

Seedling height was measured in one plant per pot per treatment with three replicates 5 d after heat exposure. Panicle length was measured from the base to the tip using a ruler. Grain yield was determined using a weighing balance at < 14% moisture content. Spikelet fertility was measured by counting the filled and unfilled grains. Percentage fertility was calculated using the computation by Sarsu (2018):

Spikelet fertility (%) = (filled grains)/(total number of reproductive sites) x 100%)

Data Analysis

Mean data were used for the analysis. One-sample *t*-tests were conducted to determine the differences between non-stress and stress trials. Analysis of Variance (ANOVA) was performed to determine which among the genotypes showed good performance. To establish relationships between parameters, mean data values were subjected to correlation analysis using R Software packages version 3.2.2. Tukey's least significant difference (LSD) at probability levels of 5% and 1% was used to compare the difference between treatments.

Pollen Fertility Assay

Pollen fertility was assessed as described by Virmani et al. (1997) and Chhun et al. (2007), with modifications. Spikelets were selected from the second or third branch from the top on the primary rachis in one plant per pot per treatment with three replicates. Three flowers with a total of 18 anthers before flowering were collected and crushed using forceps to release the pollen and were then stained with 10 µL of 1% (v/v) of I2 in 3% (v/v) KI. The fertile and infertile pollen were counted using the Infinity analyze version 6.5 (Lumenera Corporation) with 10 fields of view per replicate per variety. Fertile pollens were round and black while infertile pollens were stained yellow or light red. Percent fertility was computed using the following formula:

Pollen fertility (%) = [(no. of stained round pollen grains)/ (Total number of pollen grains)] x 100%

Fig. 2. Micrometeorological Instrument for Near Canopy Environment of Rice (MINCER) placed inside the glasshouse used in the three trials. This is the instrument used to monitor the environmental conditions inside the glasshouse.

Pollen Data Analysis

One-way ANOVA was used in comparing the pollen viability and agromorphic data among different rice varieties. One-sample *t*-test was performed to identify differences between stressed and non-stressed conditions from each genotype. Statistical analyses were conducted using R Statistical Software (R Package Version 3.5.2, R Core team 2018).

SSR Marker Search and Design

SSR loci were based on the nine QTLs distributed across chromosomes 1, 3, 4, 5, and 10 (Grospe et al. 2016; Manigbas et al. 2018). SSR primers were designed using search and design tool Genome-wide Microsatellite Analyzing Toward Application (GMATA) version 2.0 (Wang et al. 2013). Graphic GMATA mode was used for its easy interface. The input file in this study was the rice genome FASTA file with the following accessions: NC_029256.1, NC_029258.1, NC_029259.1, NC_029260.1, NC_029265.1 (NCBI 2023) for across chromosomes 1, 3, 4, 5, and 10, respectively. Parameters set were the following: primer length of 18 – 28 bp, optimal melting temperature of 57 – 60°C, optimum G + C content of 30–80%, and product size of 100–400 bp. *In silico* evaluation or e-mapping of newly designed SSRs was done using GMATA version 2.0. Ninety markers were tested using the eight genotypes in this study.

DNA Preparation and Amplification

Purified genomic DNA from leaf samples were extracted from three replicates per variety using the DNeasy Plant Mini Kit (Qiagen, Quick Strat protocol, 2016). DNA quality was estimated spectrophotometrically (Thermo Scientific NanoDrop Lite Spectrophotometer). Polymerase chain reaction (PCR) was carried out in 25 cycles with annealing temperatures of either 55°C or 58°C (T100 Thermal Cycler BioRad). PCR Promega GoTaq G2 master mix was also used for this procedure.

Poly-acrylamide Gel Electrophoresis (PAGE)

PCR-amplified products were loaded into 8% (29:1) poly-acrylamide gel with TBE buffer with a 50 bp molecular weight ladder (Vivantis NL1421). The products were electrophoresed at 100 V for 95 min. The products were viewed in Molecular Imager® Gel Doc™ XR System with Image Lab™ Software for gel imaging and analysis.

Molecular Marker Validation

For SSR analysis, 90 pairs of the newly designed SSRs were surveyed among 9 genotypes under study. The 10 primer pairs from nine determined QTL regions distributed along chromosomes 1, 3, 4, 5, and 10 for heat tolerance were tested and scored according to the resulting banding patterns among the identified heat-tolerant varieties, elite lines, and the traditional variety. Polymorphism Information Content (P_iC) was calculated using the following formula:

$$PIC = 1 - \sum_{i=1}^{n} Pi^2$$

where n is the total number of alleles detected for a given marker locus and P_i is the frequency of the i^{th} allele in the set of genotypes investigated (Botstein et al. 1980).

RESULTS AND DISCUSSSION

Agromorphic Parameters

MINCER was used to measure temperature (°C), relative humidity (RH), and dew point (°C) under glasshouse conditions. Temperature reading from the vegetative to the reproductive stage is shown in Fig. 3.

Seedling height was measured for each variety per setup in three replicates. After heat exposure at the seedling stage, it was observed that heat stress phenotypically reduced plant height (Fig. 4). The main symptom of heat stress observed was poor growth, manifested through observable yellowing of the leaves, stunted height, leaf tip burning, and leaf death in some varieties.

Although the data shows a significant reduction in plant height, it is difficult to have a conclusive generalization on the effect of heat stress on plant height since it differs depending on the variety and the experimental setup (i.e., glasshouse or field); however, it is known that heat stress mostly affects the reproductive stage.

In terms of the number of productive tillers, the study found no significant difference between stress and non-

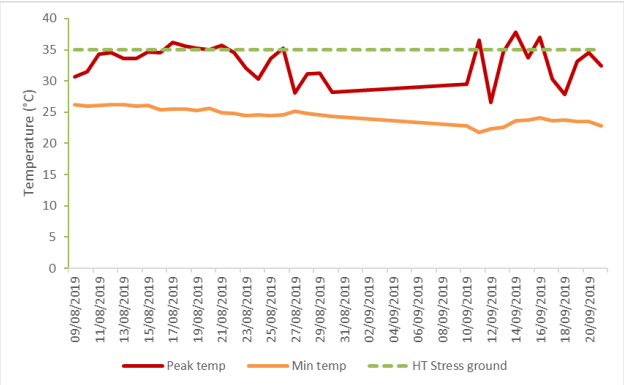

Fig. 3. Temperature readings of the 1st glasshouse trial covering the dates from the vegetative to the reproductive stage of the eight rice genotypes.

stress conditions per variety ($p > 0.05$), similar to the findings of Grospe et al. (2016). Although the number of productive tillers can also contribute to the possibility of having more tillers for panicle formation and eventually flowering and grains, these were not established parameters known to be linked or associated with heat stress, similar to plant height (Fig. 4) and panicle length (Fig. 5). However, these parameters can be used for the early selection of other types of traits that may also eventually affect heat stress. The determination of the interactions of such parameters with the various life stage of plants with conditions remains to be challenging. Heat stress tolerance, for instance, is a polygenic or recessive trait whose phenotype is influenced by more than one gene or environment. While the reproductive stage is said to be the most affected stage, heat tolerance improvement requires clear knowledge of the various mechanisms and strategies involved during stress.

In terms of panicle length (Fig. 5), the heat-tolerant varieties Dular and Nagina 22 had significant differences compared to the control. There was a reduction in length for Dular and an increase in panicle length for Nagina 22, which indicates that while both are heat-tolerant, they differ in their respective responses or adaptations to heat stress.

This stage is crucial for proper grain development as it greatly affects the potential yield of varieties. Established measurements were used to determine the spikelet fertility of the eight varieties under study (Sarsu 2018). Significant differences ($p < 0.05$) per stress condition were observed in EL92 and IR52 (Fig. 6). Comparing the EL lines, EL92 had the lowest percentage fertility while EL85 had an increase in percentage fertility during stress conditions. EL15 also showed decreased fertility under chamber conditions but was not significantly different compared to the results from its glasshouse trial.

The high-temperature stress index with corresponding values based on the quantification of reduced fertility (> 65% = highly susceptible, 25 – 65% = intermediate, < 25% = heat-tolerant) (Prasad et al. 2006; Rang et al. 2011) was used to interpret the study's results on spikelet fertility reduction as a parameter. It was observed that elite varieties EL15 and EL85 were heat-tolerant and EL92 was intermediate. Moreover, EL15 was the only elite line which showed an increase in spikelet fertility under chamber and glasshouse conditions.

Reduction in grain filling can be attributed to the possible difficulty in the capacity to mobilize carbohydrate reserves from vegetative organs under heat stress (Li et al. 2013). Yamakawa and Hakata (2010) reported that under heat stress, grain filling is disrupted because of the impaired deposition of starch and protein, eventually resulting in reduced grain yield.

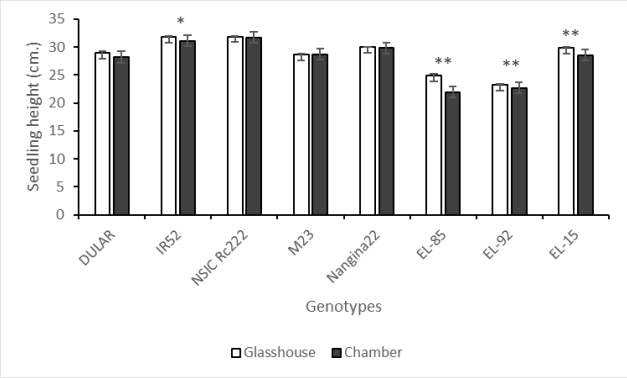

Fig. 4. Seedling height differences among the eight rice genotypes when grown in the glasshouse (unshaded bar) and growth chamber (shaded bar). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

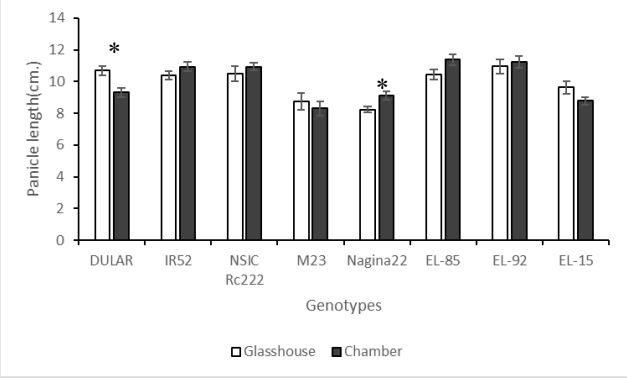

Fig. 5. Panicle length of the eight rice genotypes grown in the glasshouse (unshaded bar) and growth chamber (shaded bar) showing a significant difference ($p < 0.05$) for Nagina 22 and Dular ($p < 0.01$). Error bars represent standard errors.

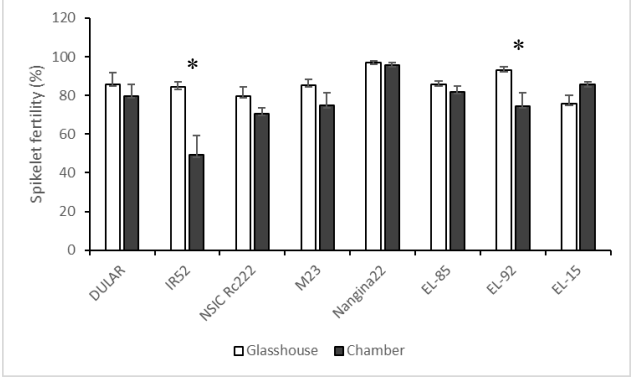

Fig. 6. Percentage fertility among the eight rice genotypes grown in the glasshouse (unshaded bar) and growth chamber (shaded bar) showing significant differences for EL-92 and IR52 ($p < 0.05$). Error bars represent standard error.

In this study, under chamber and glasshouse conditions, EL85 had the highest significant decrease in grain weight (Fig. 7). This gap between two parameters can be attributed to the grain quality of the variety under observation. One example is the difference in the size of the grain, i.e., a variety having a high percent spikelet fertility does not directly translate to having heavier grains. There could be many filled spikelets, but the grain size is small compared to other varieties.

A comparison among heat-tolerant varieties (Fig. 8) showed a significant difference only for Nagina 22. In addition, Nagina 22 had a low percentage of fertile pollen under high temperatures despite being under stress conditions as compared to Dular and NSIC Rc 222. Nagina 22, a heat-tolerant variety, showed a significant difference of $p = 0.009$ ($p < 0.01$) compared to a more controlled condition in the chamber than in the glasshouse where temperature is not controlled due to the air circulation inside. On the other hand, a known heat-tolerant variety, Dular, did not show a significant difference between conditions ($p = 0.213$). This shows that Dular seemed to have a more stable response during heat stress compared to Nagina 22, even though both are heat-tolerant varieties. Nagina 22 is, potentially, a good parent for the heat-tolerance trait, but its agromorphic performance is usually poor compared to other heat-tolerant varieties used as parents for breeding. Specifically, its known potential yield is only 2 t/ha (Bahuguna et al. 2014). Moreover, NSIC Rc 222, a high-yielding check but with no known tolerance to high temperature, can be considered as potentially tolerant because of its comparably stable performance with Dular in terms of pollen fertility.

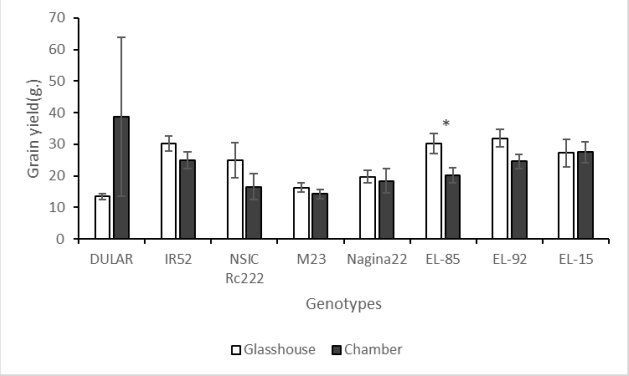

Fig. 7. Grain weight differences among the eight rice genotypes grown in the glasshouse (unshaded bar) and growth chamber (shaded bar) showing significant differences for Dular and EL85 ($p < 0.05$, $\alpha = 0.05$).

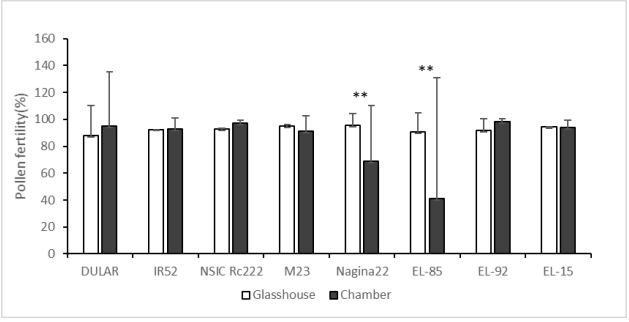

Fig. 8. Percentage pollen fertility of the eight rice genotypes grown in the glasshouse (unshaded bar) and growth chamber (shaded bar) under non-stress and heat-stress conditions.

Among the elite lines, only EL85 had the lowest percent fertile pollen (41%) during heat-stress (Fig. 8). From this, EL15 and EL92 could be the best elite lines in terms of pollen fertility. Pollen fertility does not directly translate to higher yield; however, it is important to note that EL92 had fewer fertile spikelets and a lower yield compared to EL15. Nevertheless, such a result could give vital support for these lines to be prospective donors for heat tolerance breeding since higher percent pollen fertility could entail the capacity for more viable pollen and higher chances of fertilization success. In rice, it is essential that 10 or more pollen grains germinate on the stigmata to ensure successful fertilization of the rice floret (Satake and Yoshida 1978; Matsui et al. 2001).

In selecting the phenotypes for association studies in rice, spikelet fertility has been used as the key phenotype for high-temperature (heat stress) tolerance. In most cases, finding associated traits for heat tolerance operates on the assumption that spikelet fertility is a direct tolerance indicator as tested in several studies (Cao et al. 2008; Jagadish et al. 2010; Xiao et al. 2011; Buu et al. 2014;). Traits correlated with fertility are most likely associated with high-temperature tolerance.

In Table 2, the traits that correlated with spikelet fertility were the number of productive tillers and panicle length. Although pollen fertility is not shown to be correlated with spikelet fertility, this parameter is known

Table 2. Correlation between spikelet fertility and the different key floral development traits of the eight rice genotypes.

Traits	Spikelet Fertility	
	Pearson r	*P*-value
Plant height	0.02^{ns}	0.7760
No. of productive tiller	0.21*	0.0270
Panicle length	0.32**	0.0006
Pollen fertility	0.11^{ns}	0.2450
Grain weight	0.06 ^{ns}	0.5660

** correlation is significant at the 1% level (2-tailed)

* correlation is significant at the 5% level

to contribute to grain weight and yield. A possible reason for such a result is that the number of pollens counted is only an estimate of pollen fertility and cannot accurately explain the number of pollens scattered on the stigma, thereby affecting fertilization and, ultimately, grain weight. Despite this, pollen fertility has been used in plant breeding as a screening parameter for heat tolerance (Sarsu 2018). A study by Nakamura et al. (2000) found that the number of pollen grains may be the primary factor in determining the resistance at the young microspore stage in rice cultivars. It was also pointed out in the same study that grain weight is insignificant due to the differences in the grain quality of each variety. Therefore, it is suggested that future studies re-examine the difference in the number of pollen grains per genotype for all heat-tolerant lines.

Overall, the result of the study notes that the performance of each genotype varies per parameter examined. Among the elite lines, EL15 had the highest potential as it showed increased grain weight, high percent spikelet fertility, and stable percent pollen fertility even when exposed to heat stress, thereby qualifying it to be classified as tolerant.

Validation of The SSR Markers Designed by GMATA

Using GMATA, 90 newly designed markers were selected for initial validation. These markers were tested among the panel of eight *Oryza sativa* L. varieties for both stress and non-stress conditions. All markers having amplicons produced sizes within the expected size range (Fig. 9, MK 2791), thereby supporting the claim that GMATA, as previously discussed by Wang and Wang (2016), is an efficient tool for primer design and analysis.

GMATA GFF3 file was used to view the location of the markers using the IGV (Integrative Genomics Viewer) version 2.3 (Robinson et al. 2017). The observed locations fell within the nine QTL regions of the SSR-designed markers. As the genotypes under study came from different genetic backgrounds, linkage was not possible; therefore, the 90 marker designs were already based on the nine identified QTL regions that were previously reported to be linked to heat tolerance (Grospe et al. 2016).

Molecular Marker Survey

Among the main weak points in using QTLs in plant breeding is the quality of markers used during marker-assisted selection (MAS), resulting in unreliable outcomes and ultimately leading to the erroneous perception on the failure of MAS to achieve reliable breeding improvement. Currently, most studies of MAS markers have focused on mapping population while very few studies have been conducted to examine their reliability in other genetic backgrounds. The most recent literature has honed in on the new SNP technologies; however, the most common systems used by public sector breeding programs are still traditional SSRs (Platten et al. 2019). In this study, different varieties and elite lines were selected and a traditional variety was tested across the developed SSR markers (sample gel image, Fig. 10).

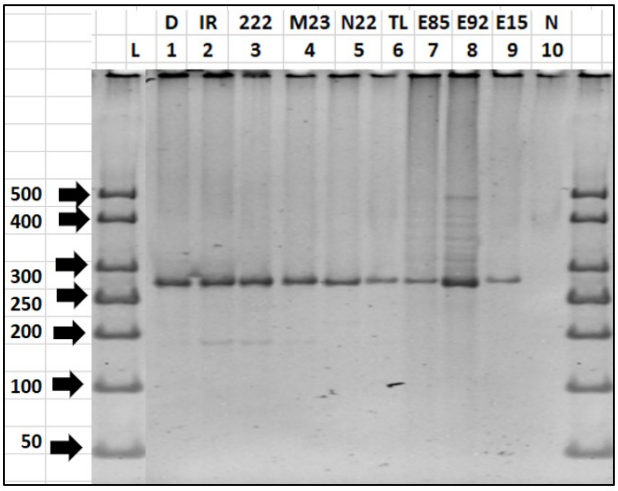

Fig. 9. Polyacrylamide gel image showing the PCR products amplified from MK2791 designed by GMATA. Lanes 1–9 represent the PCR amplicons from the DNA of rice varieties while lane 10 represents the negative check. (1) Dular, (2) IR52, (3) NSIC Rc 222, (4) Milyang 23, (5) Nagina 22, (6) EL-85, (7) EL-92, (8) EL-15, (9) Negative, (L) Vivantis 50 bp ladder.

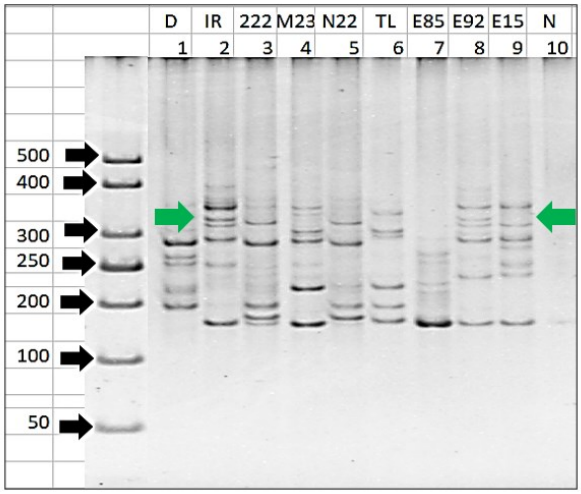

Fig. 10. Polyacrylamide gel image showing the PCR products amplified from MK2709 designed by GMATA. Lanes 1–9 represent the PCR amplicons from the DNA of rice varieties while lane 10 represents the negative check. (1) Dular, (2) IR52, (3) NSIC Rc222, (4) Milyang 23, (5) Nagina 22, (6) EL-85, (7) EL-92, (8) EL-15 (9) Negative (L) 50bp ladder. Vivantis 50 bp ladder.

From the 90 designed primers which were used for PCR with the eight rice genotypes, five markers were visually observed as polymorphic and two were identified as potentially informative markers (Table 3). For instance, marker 2709 had an absentee band (arrow) at approximately 325bp in EL15 and EL85, which is present in IR52. The same band, however, is present in EL92. This suggests that EL92 may be heat-intolerant while EL15 and EL85 may be heat-tolerant.

Marker 2503 and Marker 3066 were designed from the QTL region on chromosome 10 (Fig. 11). These showed a higher than 0.5 PiC score, thus indicating good discriminatory power (Fig. 12 and 13). This result, therefore, will not be able to determine a certain specific marker that would in turn differentiate heat-tolerant varieties from intolerant ones. However, considering the low transferability of the markers, this seems to warrant appropriate validation in future studies. Transferability and polymorphism depend on the location of the primer annealing sites and microsatellite loci in genes, respectively. High transferability means that almost all markers showed amplicons across genotypes; therefore, these may serve as effective markers for future studies (Lebedev et al. 2020) and marker-assisted breeding when any of the eight genotypes are being used for breeding heat tolerance traits.

Platten et al. (2019) attempted to address the issues surrounding the metrics and establish the criteria to identify the reliability of markers. Other studies in the case of SSRs were used in Molecular Assisted Selection (MAS) that were identified from QTL mapping populations and were applied to other genetic backgrounds — some even attempted to use SSRs in diverse germplasm panels. However, they also noted that

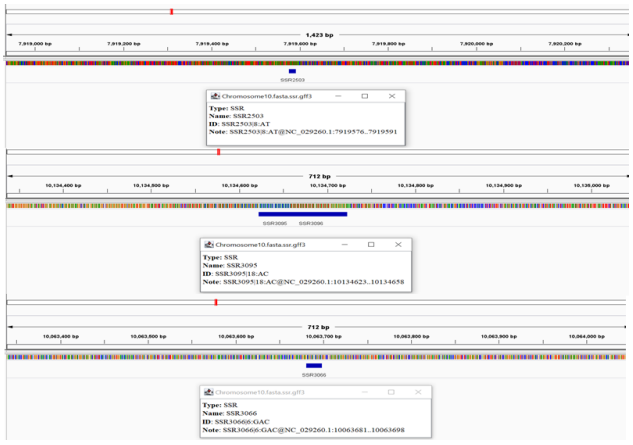

Fig. 11. Location of SSR markers designed using GMATA with IGV Software 2.3. The two SSR markers MK 2503 and MK 3066 were designed within the QTL region on chromosome 10.

Table 3. Prediction of expected size amplicons of the five potential markers using Genome-wide Microsatellite Analyzing Toward Application (GMATA) version 2.0.

Marker	Forward	Reverse	No. of Alleles	Annealing Temp (°C)	Expected Size (bp)	P_iC
MK 2503	CTGG-GAATTATTTGAGCAAGG	TCGAT-TCGGGCAAGATACTC	2	58	392	0.60
MK 3066	GGGGTCTTAGTTTCAGTCACG	TTCATTCGTTTGGGTTAGTCC	7	58	374	0.53
MK 3095	TTCATTCGTTTGGGTTAGTCC	GGGGTCTTAGTTTCAGTCACG	7	58	381	0.38
MK 2709	CCTGTT-GCTGCATGGTTTAT	CAATTCATAGGTCCTTAGCTTCTG	13	58	392	0.33
MK 2750	CTT-GGACTCTCCTTTCCTTTTC	CACGCCCA-TAGAAGTCCCTA	3	58	388	0.17

the use of SSRs in diverse germplasm requires very stringent false-positive and false-negative rates, and only very few exist where some validations of these rates had been conducted. This falls under the biological metrics which are the most important yet the most difficult to estimate. Although their established metrics provided a good framework for assessing the accuracy and reliability of any specific marker, these are, by no means, complete and perfect. The clarity metric, referring to how clearly and reliably genotyping data (bands on a gel, fluorescence signal clusters on an SNP platform or other measures) can distinguish the allelic states of the marker (Platten et al. 2019), is still said to be slightly ambiguous.

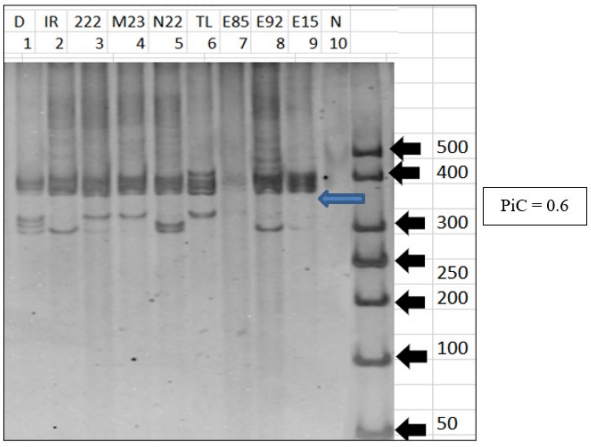

Fig. 12. Polyacrylamide gel image showing the PCR products amplified from MK2503 designed by GMATA. Lanes 1–9 represent the PCR amplicons from the DNA of rice varieties while lane 10 represents the negative check. (1) Dular, (2) IR52, (3) NSIC Rc222, (4) Milyang 23, (5) Nagina 22, (6) EL-85, (7) EL-92, (8) EL-15 (9) Negative (L) 50 bp ladder. Vivantis 50 bp ladder. Target amplicon is indicated by the arrow with PIC values.

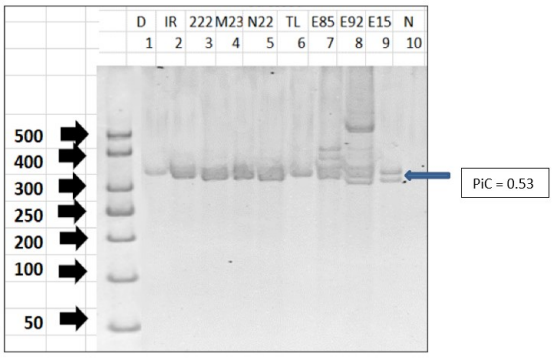

Fig. 13. Polyacrylamide gel image showing the PCR products amplified from MK3066 designed by GMATA. Lanes 1–9 represent the PCR amplicons from the DNA of rice varieties while lane 10 represents the negative check. (1) Dular, (2) IR52, (3) NSIC Rc222, (4) Milyang 23, (5) Nagina 22, (6) EL-85, (7) EL-92, (8) EL-15 (9) Negative (L) 50 bp

CONCLUSION

This study showed the specific responses of selected genotypes during heat stress under chamber and glasshouse conditions. Among the elite lines derived from heat-tolerant parents, only EL15 had the highest potential as a donor as it exhibited heat-tolerant characteristics. This line can be developed to minimize the use of resources as opposed to developing non-potential lines for heat-tolerant breeding. More researches should be conducted using varying stress conditions and validating agromorphic performance and molecular data. NSIC Rc222 can be a potential donor in developing high-yielding and tolerant varieties in the future.

Marker survey and analysis showed that the two new markers developed, MK 2503 and MK3066, have good potential in identifying the tolerant and intolerant rice varieties or elite lines due to their low transferability and good discriminatory characteristics and can be added to the list of useful markers upon the use of the eight genotypes for breeding.

Further studies using other known tolerant and intolerant rice varieties may be conducted as the banding patterns between heat stress and non-heat stress conditions vary. Standards on testing the reliability of markers including transferability, technical, biological, and breeding metrics should also be employed for proper validation.

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