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Identification of heat-tolerant rice genotypes and their molecular characterisation using SSR markers

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Abstract

The effect of high-temperature stress has a critical impact in causing reduced crop yield. The focus of the current investigation is the identification of heat-tolerant rice varieties that can alleviate the effects of stress. Among the ten genotypes evaluated across various parameters such as leaf area, dry weight, photosynthetic rate, stomatal conductance and spikelet fertility, N-22 showed superior characteristics for the grain filling parameters along with CR-Dhan 307. The variety CR-Dhan 307 with significantly higher mean pollen viability (80.23%), spikelet fertility (81.18%) and 1000 grain weight (25.45 gm) can be utilized as a heat-tolerant variety. Other genotypes Ptb-7 and CR-Dhan 202 seemed to have tolerance traits beneficial at the vegetative stage. The genotype Rajalakshmi can be characterised as heat susceptible as it had significantly lower values for all parameters. Polymorphic analysis was carried out to validate SSR markers associated with heat tolerance. The polymorphic information content (PIC) was found to be the highest for RM236 and RM6100. The SSR marker RM6100 has been validated in the current investigation to be associated with heat tolerance. As the PIC value is an indication of the ability of the marker in indicating genetic diversity, the PIC values of the 11 polymorphic markers is useful for identify heat-tolerant genotypes. The genetic diversity analysis was carried out using DendroUPGMA to establish the relationship between the genotypes. The genotypes Ptb-7 and CR-Dhan 204 were thus found to be closely related to the heat-tolerant check variety, N-22 indicating genetically related traits for tolerance to heat.

Keywords

heat stress, microsattelite markers, polymorphism, cluster analysis

Introduction

High-temperature stress critically affects the physiology of plants (1), causing debilitating losses in crop yield (2-5). Rice is an important crop that is the staple for the majority of the population in India (6). The impending rise in temperature due to climate change (7) poses a challenge to maintain the plant yield to meet the food demand of the increasing population. Rice yields are estimated to be reduced by 41% due to high temperatures by the end of the 21st century (8). Identifying regional varieties that can tolerate temperatures above 35 °C is of utmost importance in this regard. The identification of such genotypes serves the purpose of transferring their tolerance traits to high yielding cultivars through directed breeding efforts (9). The traditional breeding methods could take several years to analyse segregat-

ing characters, and therefore such research would benefit by augmenting using molecular markers. Validated molecular markers can rapidly identify tolerance traits at the gene level, thereby reducing the time required to develop high yielding tolerant cultivars (10). The objective of the present investigation is to identify heat-tolerant genotypes through the evaluation of physiological and yield-related traits. Simple sequence repeats (SSR) are widely used markers in marker-assisted selection (MAS) due to to easy availability, comparatively cheaper than others and they require a relatively simple technique with a higher polymorphism rate (11). A study conducted with SSR markers and associated agronomic and yield related traits was able to identify 45 candidate genes related to nine makers and significantly associated with six traits under heat stress conditions (12). SSR markers showing polymorphism between the tolerant and susceptible genotypes were validated in the current study.

Materials and Methods

The study was conducted at the College of Agriculture, Thiruvananthapuram, located at 8.44 °N, 76.99 °E in the southern part of India. The experiment was conducted in 2021 from February to May with an average maximum temperature ranging from 31-34 °C. The plants were placed in both open field conditions and in a 200 m^2 poly-house where the temperature was recorded to be about 8-10 °C higher than the ambient conditions giving an effective temperature ranging from 38-42 °C.

The varieties selected for evaluation were Ajay, Apo, CR-Dhan 202, CR-Dhan 204, CR-Dhan 305, CR-Dhan 307, CR -Dhan 701, Jyothi, Nagina-22, Ptb-7 and Rajalakshmi (Table 1). The plants were grown in pots of size 25 × 12 cm with two plants per pot. The experiment was conducted in the completely randomized design (CRD) with three replications maintained for each variety. The fertiliser, pest and disease management was practised as recommended by the Kerala Agricultural University (13). The plants were maintained with continuous irrigation without any drought conditions. The plants were grown under ambient conditions until the maximum tillering stage, after which the plants of the high-temperature treatments were moved to the polyhouse, where heat stress was imposed until harvesting. The statistical analysis was done using statistical package GRAPES 1.0.0 (14). The critical difference was calculated at 95 % level of confidence.

Table 1. List of rice genotypes and their parentage used in the study.

S.No	Genotypes	Parentage or Pedigree
V1	N22 (Nagina)	A selection from Rajbhog
V2	Apo	IR55423-01
V3	Ptb 7 (Parambuvattan)	Landrace
V4	Ajay	Hybrid- CRMS 31A/IR-42266-29-3-R
V5	Rajalakshmi	Hybrid-CRMS 32A/IR-42266-29-3R
V6	CR Dhan 202	IRRI 148/IR 78877-208-B-1-1
V7	CR Dhan 204	IRRI 76569-25999---1-2-1/ CT 6510-24-1-2
V8	CR Dhan 305	R 77080-B34-3/IRRI 123 2
V9	CR Dhan 307 (Maudamani)	Dandi / Naveen // Dandi
V10	CR Dhan 701	CRMS 31A/CRL-22R
V11	Jyothi (PTB39)	Hybrid derivative-PTB 10 X IR8

Observations recorded

All the observations were taken at the flowering stage. The leaf area was measured by taking a mean of 6 leaves from random hills. The area of each leaf was calculated by multiplying the length of the leaf with the width at the centre of the leaf and the leaf area constant of 0.75 (15). The root and shoot dry weights were calculated after drying the respective plant parts until a constant weight was obtained consecutively and expressed in grams. The photosynthetic rate and stomatal conductance were measured using an Infra-red gas analyser (Li-Cor 6400XT, USA). The viability of the pollen was observed by staining the pollen grains with 1% iodine-Potassium iodide solution. The pollen viability (%) was measured by the formula:

$$\frac{\text{The number of stained pollen grains}}{\text{total number of pollen grains}} \text{ X } 100$$

The spikelet fertility and 1000 grain weight were calculated at the harvest stage. The spikelet fertility percentage was dividing the number of filled grains by the total number of grains in a panicle, the whole multiplied by 100. The weight of 1000 grains was measured and expressed in grams as the 1000 grain weight.

The DNA was isolated using the CTAB method (16), wherein plant samples were ground in liquid nitrogen and homogenised using CTAB buffer and extracted using chloroform: isoamyl alcohol (24:1). The DNA was quantified by the ratio of absorbance at 260/280 nm. The quality of DNA was checked by running on 0.8% agarose gel.

PCR amplification using SSR primers

The PCR reaction was performed in a 20 µl reaction mixture which consisted of genomic DNA (25 ng/µl) - 2.0 µl, 10X Taq assay buffer A - 2.0 µl, dNTPs mix (10 µl each) - 1.5 µl, Taq DNA polymerase (1U) - 0.3 µl, forward primer (10 pM) - 0.75 µl, reverse primer (10 pM) - 0.75 µl and the remaining was made up with autoclaved distilled water (12.7 µl). The thermal cycling was carried out with the following conditions: 1) Initial denaturation—94 °C for 3 min 2) Denaturation—94 °C for 1 min 3) Primer annealing—53 °C to 55 °C for 1 min 4) Primer extension—72 °C for 1 min 5) Final extension—72 °C for 5 min 6) Incubation—4 °C for infinity to hold the sample. Steps 2 to 4 were repeated for 35 cycles.

Detection of polymorphism between the genotypes using SSR markers

The PCR screening was carried out using 30 reported microsatellite SSR markers linked to drought, salinity and

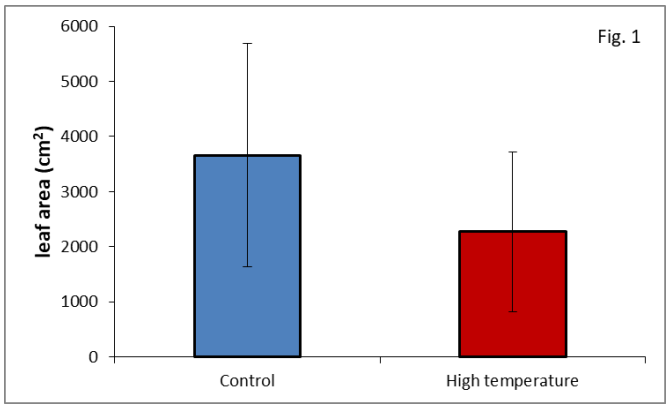
temperature. The PCR products were separated on agarose gel along with the marker (100bp ladder) and 1X TBE buffer. Ethidium bromide was used for staining purposes. The gel profile was visualised using (Syngene G box documentation system). The documented SSR profiles were carefully examined for the polymorphism in the banding pattern between the rice genotypes.

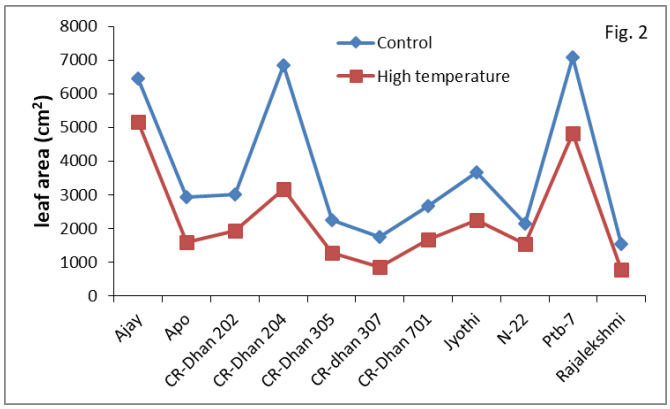
Results and Discussion

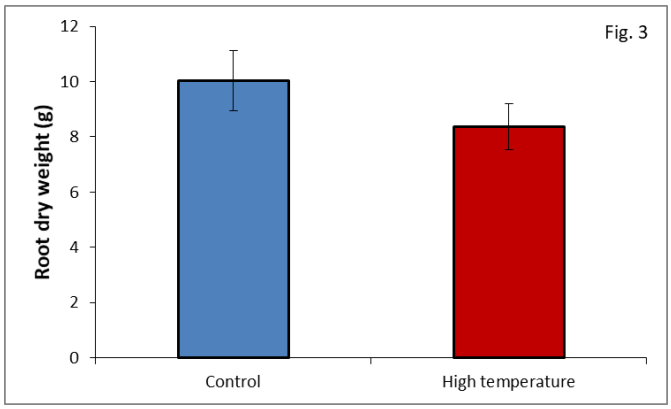
Leaf area

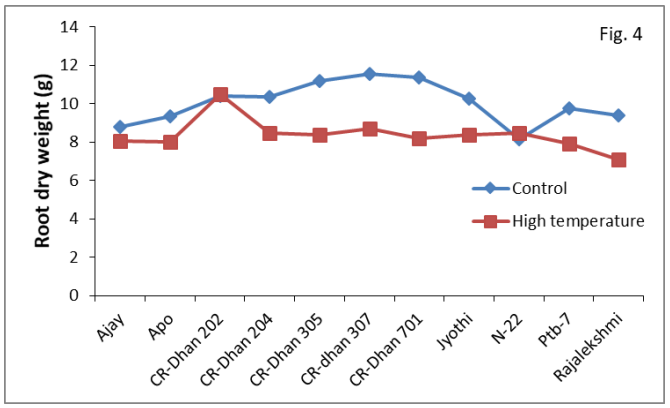
The leaf area of all genotypes significantly decreased when they were subjected to high-temperature stress. The mean leaf area of genotypes subjected to stress was 2275.46 cm^2 compared to control/open conditions (3661.99 cm^2) (Fig. 1). Among the genotypes (Table 2), Ptb-7 showed the highest mean leaf area (5947.9 cm^2), followed by Ajay (5795.91 cm^2) and CR-Dhan 204 (5004.9 cm^2). The least leaf area was recorded in CR-Dhan 307 (1294.65 cm^2), followed by Rajalakshmi (1158.9 cm^2). The interaction of genotype and stress factors (Fig. 2) revealed that Ptb-7 under control conditions recorded the highest leaf area of 7077.66 cm^2, followed by CR-Dhan 204 (6837.13 cm^2). Under high-temperature stress, the genotype Ajay recorded the highest leaf area (5153.53 cm^2), followed by Ptb-7 (4818.13 cm^2). The leaf area was the lowest in CR-Dhan 307 (847.76 cm^2) and Rajalakshmi (774.76 cm^2), both on par and under high-temperature stress.

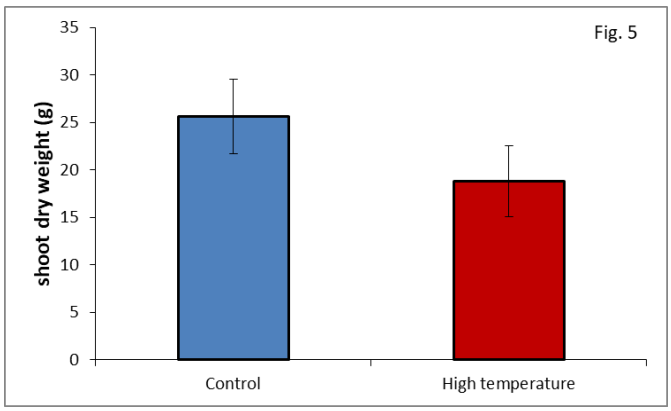
Greater leaf area indicates better growth and increased area for photosynthesis and assimilation. Ptb-7 and Ajay, with their higher leaf area, have an advantage in this regard and are better suited to withstand stress compared to the genotype Rajalakshmi which had a significantly decreased leaf area. The genotypes Ptb-7 and Ajay can be characterised as tolerant as they maintained higher leaf area even under higher temperatures. The varieties CR -Dhan 307 and Rajalakshmi had significantly reduced leaf area and, therefore, may be characterised as susceptible to high-temperature stress. The leaf area was reported to be significantly reduced in rice genotypes subjected to heat stress in an earlier study (17), which noted that it was correlated to a reduction in the assimilation of photosynthates. A positive correlation of the leaf area index with

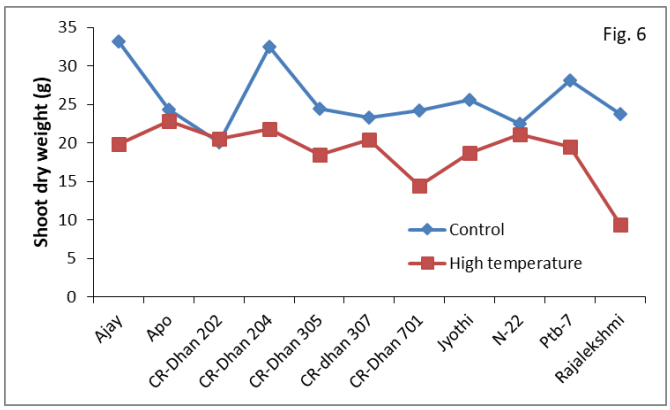


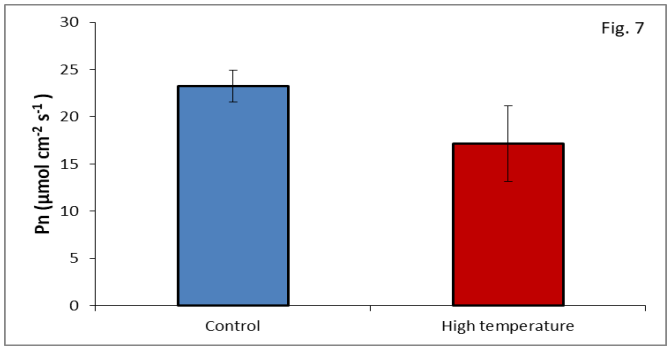


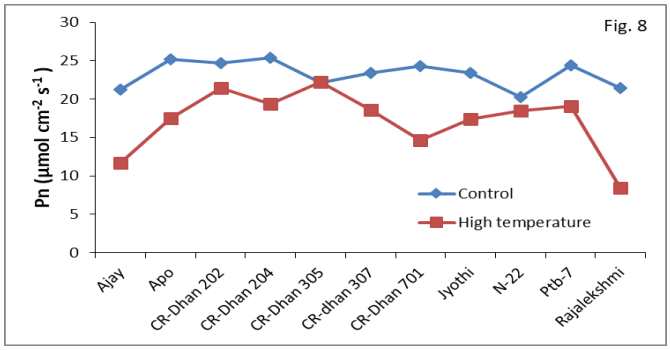


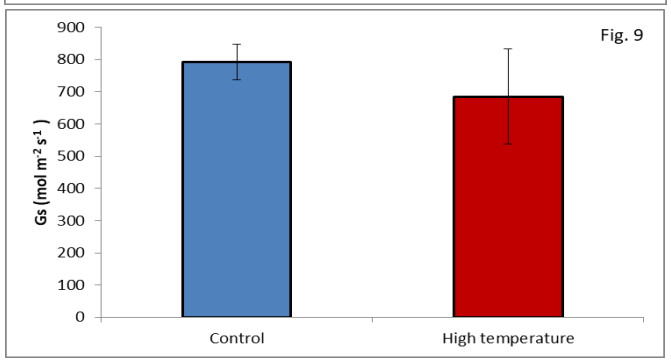


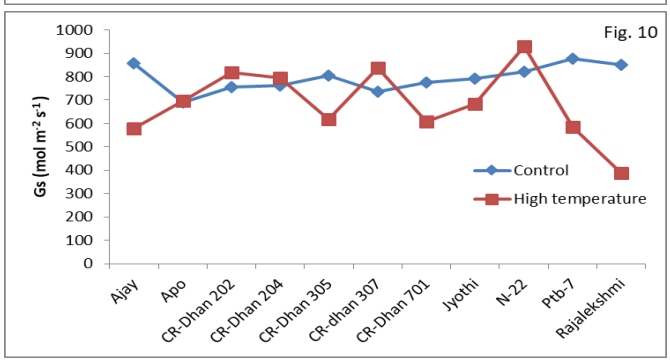


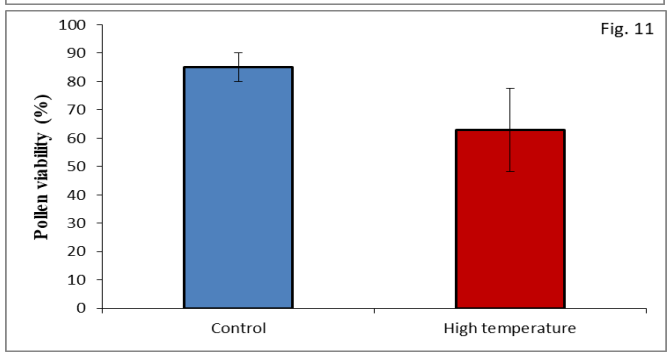


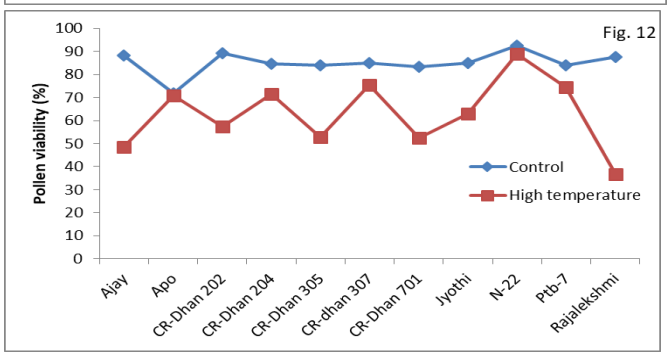


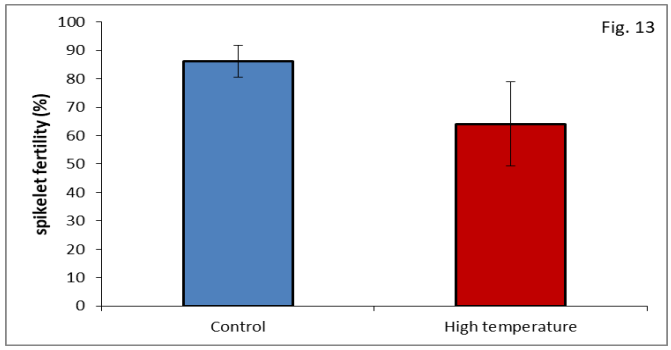


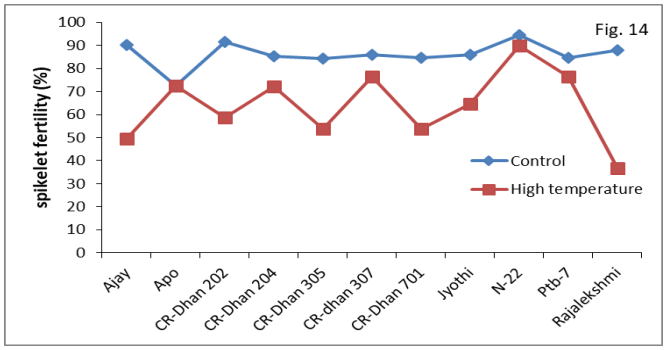


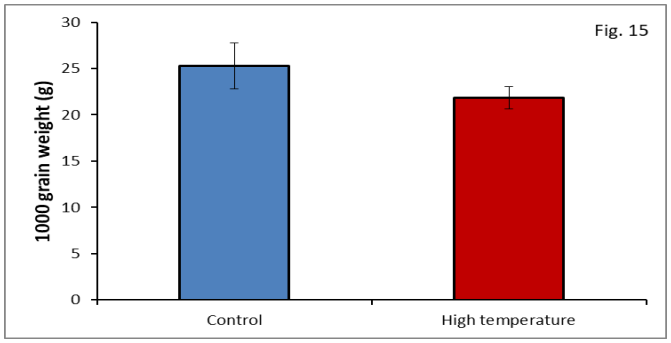


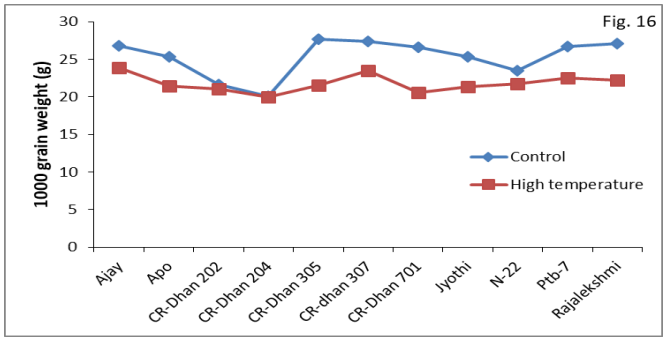












*The mean of all genotypes under control conditions and high temperature conditions has been presented in **Fig. 1** (Leaf area), **Fig. 3** (root dry weight), **Fig. 5** (shoot dry weight), **Fig. 7** (Photosynthetic rate - Pn), **Fig. 9** (Stomatal conductance - Gs), **Fig. 11** (Pollen viability), **Fig. 13** (Spikelet fertility) and **Fig. 15** (1000 grain weight).*

*The mean of each genotype under control conditions and high temperature conditions has been presented in **Fig. 2** (Leaf area), **Fig. 4** (root dry weight), **Fig. 6** (shoot dry weight), **Fig. 8** (Photosynthetic rate - Pn), **Fig. 10** (Stomatal conductance - Gs), **Fig. 12** (Pollen viability), **Fig. 14** (Spikelet fertility) and **Fig. 16** (1000 grain weight).*

Fig. (1-16): The mean of genotypes under control and high temperature conditions

Table 2. The mean of the genotypes averaged across control and high-temperature stress. The parameters were analysed statistically ($p<0.05$). The genotypes with similar letters are not significant. Leaf area (cm^2), RDW- Root dry weight (gm), SDW – Shoot dry weight (gm), Pn- photosynthetic rate (µmol cm^{-2} s^{-1}), Gs – stomatal conductance (mol m^{-2} s^{-1}), pollen viability (%), spikelet fertility (%) and 1000 grain weight (gm). C.D.- critical difference; S.E. (m) – standard error (mean)

Genotype	Leaf Area	RDW	SDW	Pn	Gs	Pollen via-bility	spikelet fertility	1000 grain weight
Ajay	5795.917^{b}	8.417^{efg}	26.517^{b}	16.433^{h}	717^{d}	68.4^{f}	69.933^{f}	25.333^{a}
Apo	2254.467^{f}	8.667^{ef}	23.567^{c}	21.367^{d}	692.517^{e}	71.383^{e}	72.467^{e}	23.417^{c}
CR-Dhan 202	2481.45^{e}	10.433^{a}	20.3^{f}	23.067^{a}	786.417^{b}	73.35^{d}	75^{d}	21.35^{e}
CR-Dhan 204	5004.9^{c}	9.417^{cd}	27.167^{a}	22.333^{b}	778.817^{b}	78.083^{c}	78.767^{c}	20.05^{f}
CR-Dhan 305	1751.733^{g}	9.767^{bc}	21.433^{e}	22.217^{b}	711.8^{d}	68.45^{f}	69.033^{f}	24.633^{b}
CR-dhan 307	1294.65^{h}	10.117^{ab}	21.833^{de}	20.967^{e}	786.367^{b}	80.233^{b}	81.183^{b}	25.45^{a}
CR-Dhan 701	2157.85^{f}	9.767^{bc}	19.283^{g}	19.433^{g}	691.783^{e}	67.783^{f}	69.15^{f}	23.617^{c}
Jyothi	2959.6^{d}	9.3^{d}	22.117^{d}	20.4^{f}	738.333^{c}	73.9^{d}	75.367^{d}	23.4^{c}
N-22	1848.617^{g}	8.3f^{g}	21.767^{de}	19.35^{g}	875.35^{a}	90.85^{a}	92.317^{a}	22.633^{d}
Ptb-7	5947.9^{a}	8.833^{e}	23.767^{c}	21.733^{c}	731.1^{c}	79.367^{b}	80.617^{b}	24.6^{b}
Rajalakshmi	1158.9^{i}	8.233^{g}	16.567^{h}	14.933^{i}	618.95^{f}	62.117^{g}	62.333^{g}	24.667^{b}
Mean	2968.72	9.20	22.21	20.20	738.94	73.99	75.1	23.55
C.D.	97.006	0.418	0.484	0.243	8.877	0.9	1.346	0.572
S.E.(m)	34.033	0.147	0.17	0.085	3.115	0.316	0.472	0.201

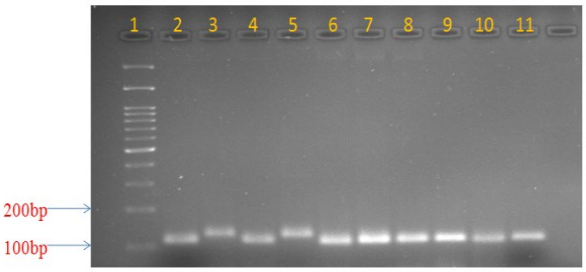


Figure 17 – RM1003

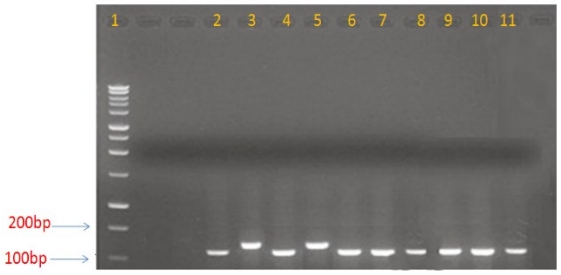


Figure 18 – RM167

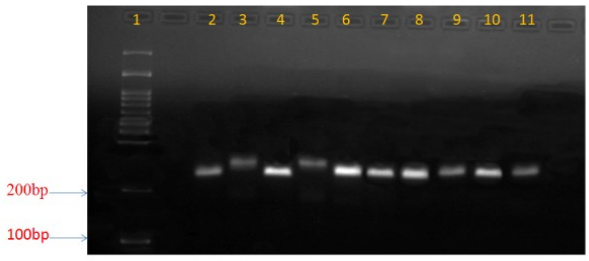


Figure 19 – RM474

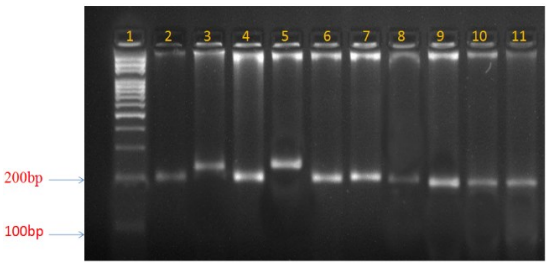


Figure 20 - RM303

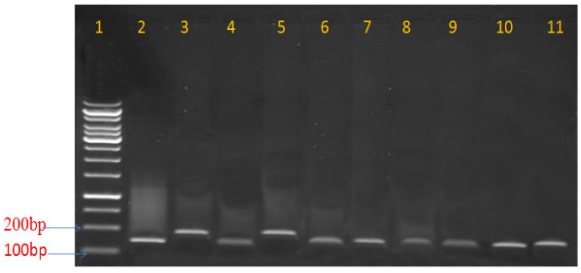


Figure 21 - RM302

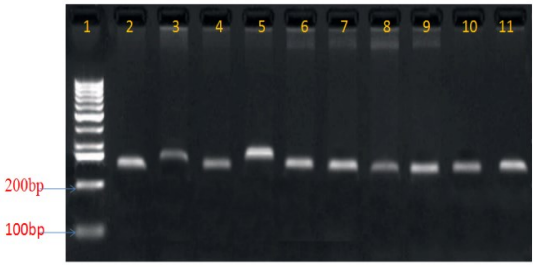


Figure 22 - RM484

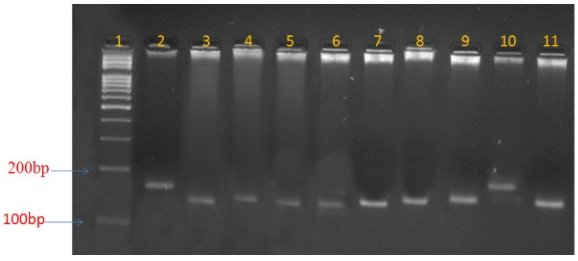


Figure 23 - RM7117

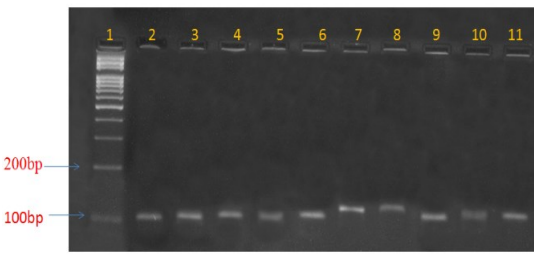


Figure 24 - RM271

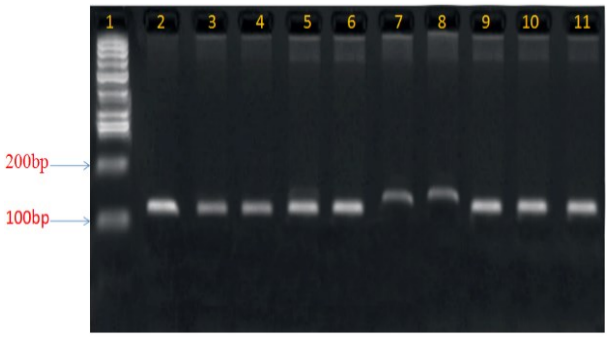


Figure 25 - RM525

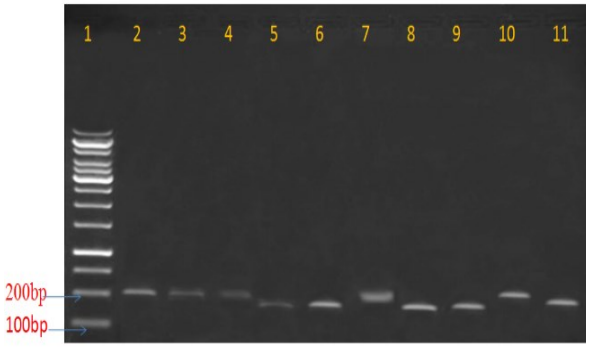


Figure 26 - RM236

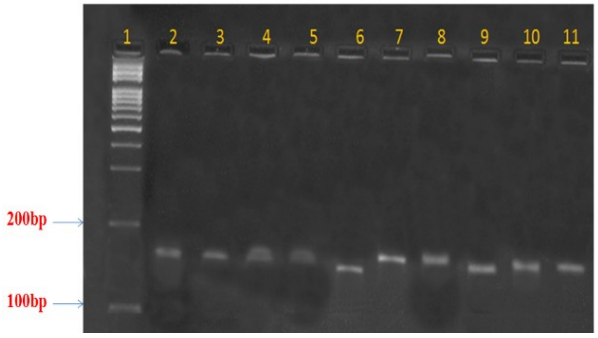


Figure 27 - RM6100

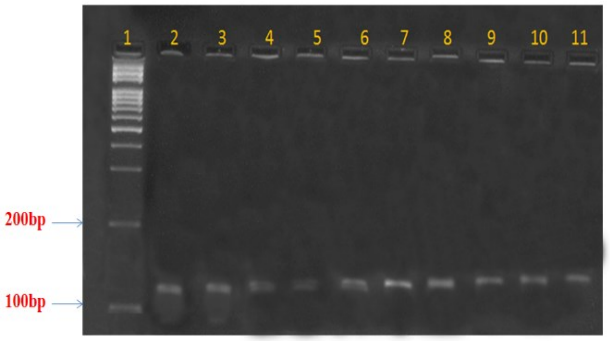


Figure 28 - RM112

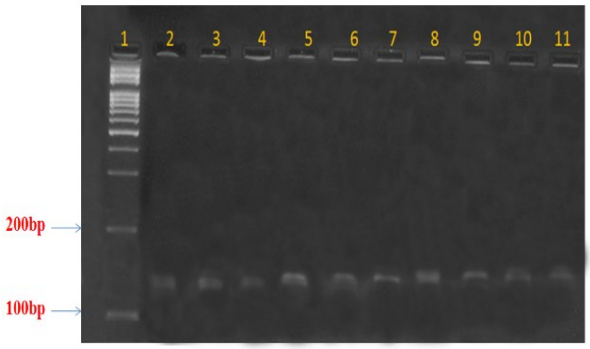


Figure 29 - RM80

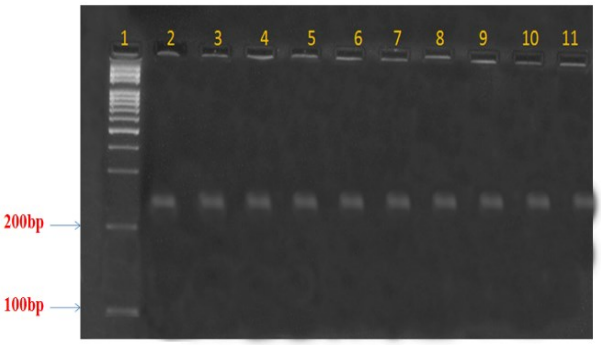


Figure 30 - RM527

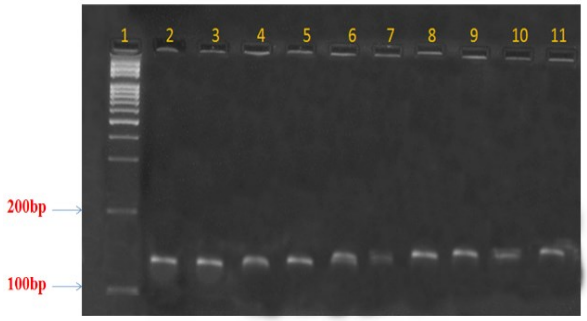


Figure 31 - RM255

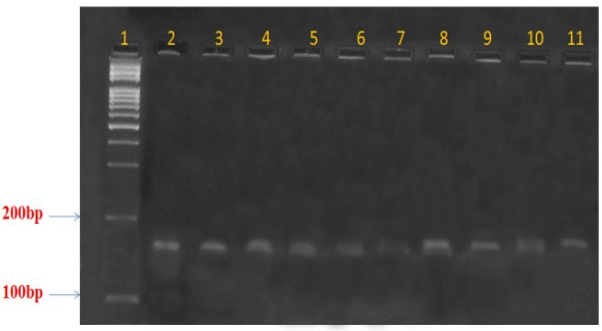


Figure 32 - RM6

Fig. (17-32): Amplification pattern of 10 rice genotypes obtained by SSR markers

(Lane 1- 100bp ladder, Lane 2- PTB 7, Lane 3- CR-Dhan 202, Lane 4- CR-Dhan 204, Lane 5- CR-Dhan 305, Lane 6- CR-Dhan 307, Lane 7- CR-Dhan 701, Lane 8- Ajay, Lane 9- APO, Lane 10- Nagina 22, Lane 11- Rajalakshmi)

Root Dry Weight (RDW)

The root dry weight presented in Fig. 3 and 4 and Table 3 showed significant differences between treatments and genotypes. The mean root dry weight under high temperature was 8.37 gm while it was 10.03 gm under control (Fig. 3). The mean root dry weight of the genotypes (Table 1) was highest in CR-Dhan 202 (10.43 gm) followed by CR-Dhan 307 (10.117 gm), which were on par with one another, while the lowest was recorded in N-22 and Rajalakshmi, which were on par with 8.3 gm and 8.23 gm respectively. The interaction data on treatment × genotypes (Fig. 4) revealed that CR-Dhan 307 (11.53 gm), CR-Dhan 701 (11.36 gm) and CR-Dhan 305 (11.16 gm), which were on par with one another under control, recorded the highest root dry weight. CR-Dhan 202 genotype recorded the highest root dry weight of 10.46 gm under stress conditions. The least root dry weight was recorded in Apo (8 gm), Ptb-7 (7.9 gm) and Rajalakshmi (7.1 gm) under high-temperature treatment.

The root dry weight of CR-Dhan 202 and CR-Dhan 307 was highest among all the genotypes, indicating better growth and increased nutrient translocation capacity. Even

grain yield and photosynthetic rate under high-temperature stress was noted in a similar study (18). Under high-temperature stress, the two genotypes attributed to decreased partitioning and accumulation of photo-assimilates in the roots or increased rates of transpiration (19). A similar study indicated that genotypes with greater root dry matter had increased thermo-tolerance (20).

Shoot Dry Weight (SDW)

The mean shoot dry weight of genotypes under high temperature was significantly lower at 18.79 g than the control (25.62 gm) (Fig. 5). Among the genotypes (Table 2), CR-Dhan 204 recorded the highest shoot dry weight of 27.16 gm followed by Ajay (26.51 gm), while the least was recorded in CR-Dhan 701 (19.28 gm) and Rajalakshmi (16.5 gm). The interaction of factors (Fig. 6) reveals that Ajay and CR-Dhan 204 under control recorded the highest shoot dry weight of 33.2 gm and 32.53 gm. Under high-temperature stress, Apo recorded the highest shoot dry weight of 22.76 gm followed by CR-Dhan 204 (21.8 gm). The lowest shoot dry weight was recorded in CR-Dhan 701 (14.4 gm) and Rajalakshmi (9.36 gm).

The shoot dry weight, which is a stable indicator of growth and development, was the highest in CR-Dhan 204 and Ajay, while the least was in the genotype Rajaleskmi. However, in the interaction of genotype × stress factors, only CR-Dhan 204 could maintain its higher shoot dry weight under high temperatures. Under stress conditions, the percentage decrease in shoot dry weight was steep and was significantly reduced in the genotypes CR-Dhan 701 (- 40.41%) and Rajalakshmi (- 60.58%). A study on wheat genotypes reported that varieties with higher shoot dry weight were better able to withstand high-temperature stress (21). Decreased shoot dry matter accumulation in tomatoes under high temperature was found to be significantly correlated with increased production of reactive oxygen species leading to oxidative damage (22).

Photosynthetic rate

The photosynthetic rate (Fig. 7) was significantly lower under high-temperature treatment (17.15 µmol cm^{-2} s^{-1}) compared to the control (23.25 µmol cm^{-2} s^{-1}). Among genotypes (Table 2), CR-Dhan 2022 (23.06 µmol cm^{-2} s^{-1}) recorded the highest photosynthetic rate, followed by CR-Dhan 204 (22.33 µmol cm^{-2} s^{-1}) and CR-Dhan 305 (22.21 µmol cm^{-2} s^{-1}), which was on par. The lowest photosynthetic rate was recorded in genotypes Ajay (16.43 µmol cm^{-2} s^{-1}) and Rajalakshmi (14.93 µmol cm^{-2} s^{-1}). The interaction data (Fig. 8) revealed that under control treatment, the genotypes CR-Dhan 204 and Apo were on par with photosynthetic rates of 25.33 µmol cm^{-2} s^{-1} and 25.2 µmol cm^{-2} s^{-1} followed by CR-Dhan 202 (24.73 µmol cm^{-2} s^{-1}). Under the stress treatments, the highest photosynthetic rate was recorded in the genotype CR-Dhan 305 (22.26 µmol cm^{-2} s^{-1}) followed by CR-Dhan 202 (21.4 µmol cm^{-2} s^{-1}). The genotypes Ajay (11.63 µmol cm^{-2} s^{-1}) and Rajalakshmi (8.43 µmol cm^{-2} s^{-1}) recorded the least photosynthetic rate under high-temperature treatment.

The varieties CR-Dhan 204 and Apo recorded the highest photosynthetic rate under control conditions. However, under high-temperature stress, the highest photosynthetic rate was recorded in CR-Dhan 305 and CR-Dhan 202. The genotype Apo recorded a reduction of 30.43% under stress, while the highest reduction in photosynthetic rate due to high temperature was recorded in Rajalakshmi with a 60.67% reduction. The photosynthetic rate of plants at high temperatures is lowered mainly due to the deactivation of Rubisco activase enzyme due to the excessive production of reactive oxygen species (23). Heat stress at the grain filling stage decreased the photosynthetic rate, resulting in the limitation of dry matter translocation into the grains (24).

Stomatal Conductance

The stomatal conductance under high-temperature treatment was significantly reduced (685.12 mol m^{-2} s^{-1}) with respect to the control (792.77 mol m^{-2} s^{-1}) (Fig. 9). Among the genotypes (Table 2), N-22 was found to have the highest stomatal conductance of 875.35 mol m^{-2} s^{-1}, followed by CR-Dhan 202 (786.41 mol m^{-2} s^{-1}), CR-Dhan 307 (786.36 mol m^{-2} s^{-1}) and CR-Dhan 204 (778.81 mol m^{-2} s^{-1}), which were on-par with one another. The least stomatal conductance was recorded in Rajalakshmi (618.95 mol m^{-2} s^{-1}). The data for interaction (Fig. 10) between treatment and genotype revealed that N-22 recorded the highest stomatal conductance of 930.03 mol m^{-2} s^{-1} under high-temperature treatment followed by CR-Dhan 307 (836.67 mol m^{-2} s^{-1}). Under control treatment, Ptb-7 recorded the highest stomatal conductance (876.33 mol m^{-2} s^{-1}).

The variety N-22 exhibited the highest stomatal conductance under high-temperature stress. This is also validated in an earlier study (25), which reported higher mean stomatal conductance in the variety N-22. Apart from N-22, the genotypes CR-Dhan 307, CR-Dhan 202 and CR-Dhan 204 recorded significantly higher stomatal conductance under high temperatures compared to their respective control treatments. The varieties exhibiting higher stomatal conductance under high-temperature stress are tolerant as increased stomatal conductance increases the transpiration rate allowing the plant tissue temperature to be lowered, thereby alleviating the oxidative stress. The temperature of the individual organs such as leaves or spikelets is regulated mainly by cooling through transpiration (26). The genotypes Ptb-7, Ajay and Rajalakshmi have significantly reduced stomatal conductance under stress conditions indicating their susceptibility. Stomatal conductance has been reported to be genetically determined in cotton and has been strongly correlated to thermotolerance or heat avoidance mechanism (27).

Pollen Viability

High-temperature stress significantly decreased the pollen viability (62.92%) compared to control (85.06%) (Fig. 11). There was a significant difference among the genotypes (Table 2). The mean of high temperature and normal conditions revealed that N-22 recorded the highest pollen viability (90.85%), followed by CR-Dhan 307 (80.23%) and Ptb-7 (79.36%) which were on par, while the least was recorded in Rajalakshmi (62.11%). The lowest pollen viability

percentage was recorded in Ajay (48.67%) and Rajalakshmi (36.56%) under high-temperature stress.

The variety N-22 showed the highest pollen viability with only a 3.6% reduction between the control and high-temperature treatments. The greatest reduction in pollen viability under high-temperature stress was observed in Ajay (-36.46%) and Rajalakshmi (-51.11%). A similar study (28) also reported a sharp decrease in pollen viability by 37.52% in plants subjected to heat stress. The impairment of starch mobilisation from the leaves to the reproductive organs due to heat stress is the major cause of the failure of pollen to germinate (29). The sugar metabolism is critically affected by heat stress in the anther and pollen (30). The lower assimilate availability is attributed to the impairment of enzyme activity involved in starch metabolism (31). Pollen viability is an important contributing factor for spikelet fertility and successful grain filling, leading to increased yield.

Spikelet fertility

The data presented on spikelet fertility showed a significant difference between genotypes and treatments. The spikelet fertility of the panicles (Fig. 13) under control conditions was higher (86.13%) compared to those under high -temperature treatments (64.07%). Among the genotypes (Table 2), N-22 recorded the highest spikelet fertility with 92.31%, followed by CR-Dhan 307 (81.18%) and Ptb-7 (80.61%). The least spikelet fertility was recorded in the genotype Rajalakshmi (62.33%). The interaction data (Fig. 14) revealed that N-22 under control maintained the highest spikelet fertility percentage at 94.56%, followed by CR-Dhan 202 (91.4%) and Ajay (90.33%) under control conditions and N-22 under high-temperature treatment (90.06%), the three of which were on par. The least spikelet fertility of 36.67% was recorded in Rajalakshmi under high-temperature treatment.

The spikelet fertility of the genotype N-22 was the highest among all the genotypes subjected to high-temperature stress with only a 4.5% reduction compared to its control. Compared to their control, the other genotypes that could maintain relatively high spikelet fertility under stress were Ptb-7 and CR-Dhan 307, reducing 8.03% and 9.3% respectively. In another study (32), high-temperature stress was found to significantly reduce the spikelet fertility (-65.06%) in the susceptible rice genotype. Several factors are involved in the sterility of spikelets, such as non-viability of pollen, inhibition of pollen tube elongation (33), impairment of starch and sucrose metabolism in grain filling, anther indehiscence and deterioration of ovarian tissue (34). Spikelet fertility is a clear indicator of thermo-tolerance as it is an important contributing factor that decides the ultimate yield of the crop.

1000 grain weight

The 1000 grain weight (Fig. 15) of genotypes under high temperature (21.82 gm) is significantly lower than control (25.29 gm). The statistical analysis of genotypes (Table 2) reveals that CR-Dhan 307 (25.45 gm) and Ajay (25.33 gm) were on-par and highest and on-par. The least 1000 grain weight was recorded in CR-Dhan 202 (21.35 gm) and CR-Dhan 204 (20.05 gm). The interaction of the factors, i.e. genotype and treatment (Fig. 16), shows that CR-Dhan 305 recorded the highest 1000 grain weight of 27.7 gm under control conditions followed by CR-Dhan 307 (27.36 gm) and Rajalakshmi (27.13 gm), also under control conditions and was on-par. The least 1000 grain weight was recorded in CR-Dhan 204 under control (20.1 gm) and high temperature (20 gm) conditions. There was no significant difference between the treatments in this genotype.

The grain weight of a variety is a genetic character that the environment can strongly influence, especially high-temperature stress. In this regard, N-22 seems to be tolerant as the 1000 grain weight reduction in high-temperature stress was only -7.4%, followed by Ajay with -10.48% reduction and CR-Dhan 307 (-13.99%). A drastic reduction in the grain yield per plant by almost 90% was reported under high-temperature stress (35). The grain weight is a result of the accumulation of photo-assimilates that are translocated from the actively photosynthesising flag leaf and storage organs such as stems. Starch hydrolysing enzymes such as starch synthase (SS), invertases (INVs) and enzymes involved in sucrose metabolism such as Sucrose Synthase (*SuSy*) play a significant role in ensuring the enhanced accumulation of assimilates under heat stress (36).

Correlation Analysis

The correlation (Table 3) analysis conducted between the various parameters revealed that the 1000 grain weight, the ultimate yield indicator, was positively correlated with stomatal conductance, spikelet fertility, photosynthetic

Table 3. Correlation analysis of various parameters under high-temperature stress. Gs- stomatal conductance, Pn – photosynthetic rate. *** indicates correlation is significant at 0.001 level, ** indicates correlation is significant at 0.01 level, * indicates correlation is significant at 0.05 level.

	(Gs)	1000 grain weight	Spikelet fertility	(Pn)	Leaf Area	Root dry wt.	Shoot Dry wt.	Pollen viability
(Gs)	1	0.312*	0.794***	0.623***	0.175	0.462***	0.671***	0.798***
1000 grain weight	0.312*	1	0.428***	0.344**	0.149	0.479***	0.419***	0.442***
Spikelet fertility	0.794***	0.428***	1	0.712***	0.284*	0.497***	0.712***	0.996***
(Pn)	0.623***	0.344**	0.712***	1	0.303*	0.73***	0.708***	0.715***
Leaf Area	0.175	0.149	0.284*	0.303*	1	0.138	0.676***	0.28*
Root dry wt.	0.462***	0.479***	0.497***	0.73***	0.138	1	0.489***	0.505***
Shoot Dry wt.	0.671***	0.419***	0.712***	0.708***	0.676***	0.489***	1	0.715***
Pollen viability	0.798***	0.442***	0.996***	0.715***	0.28*	0.505***	0.715***	1

rate, shoot and root dry weight and pollen viability. No significant correlation was found between leaf area and 1000 grain weight. However, leaf area was significantly correlated with dry shoot weight at 0.001 level and spikelet fertility, photosynthetic rate and pollen viability at 0.05 level.

PCR amplification using SSR primers

PCR reactions were performed using the selected primer by providing appropriate PCR conditions. Out of fifty primers, 11 of them showed polymorphism in 3.5% agarose gel electrophoresis. RM1003, RM167, RM474, RM303, RM302, RM484, RM7117, RM271, RM525, RM6100, and RM236 were the polymorphic markers. The size of the polymorphic bands amplified by each marker has been mentioned in Table 4. The banding pattern of amplified products with RM1003, RM167, RM474, RM303, RM302, RM484, RM7117, RM271, RM525, RM6100 and RM236 in 3.5% agarose gel is shown in figure 17-27. The monomorphic primers are presented in Fig. 28-32.

Table 4. PIC values of primers.

Sl.No.	Primer	PIC value
1	RM1003	0.66
2	RM167	0.66
3	RM474	0.66
4	RM7117	0.66
5	RM271	0.66
6	RM303	0.66
7	RM302	0.66
8	RM484	0.66
9	RM525	0.66
10	RM236	0.75
11	RM6100	0.71

Table 5. Average length of band amplified by SSR markers.

S.no.	Marker	Size of band (bp)
1)	RM1003	~128
2)	RM167	~111
3)	RM474	~252
4)	RM303	~200
5)	RM302	~156
6)	RM484	~299
7)	RM7117	~158
8)	RM271	~101
9)	RM525	~131
10)	RM236	~191
11)	RM6100	~152

SSR marker polymorphism

SSR markers are valuable as genetic markers because they detect high levels of allelic diversity, co-dominant, easy and economically assayed by PCR, easily automated (37), abundance, and even genomic distribution (38) and high level of polymorphism (39). It has an average polymorphism at least 1.5 times higher than AFLP and RAPD markers (40). SSRs are highly polymorphic even between closely related lines (41). The polymorphism in SSR could be due to a change in the SSR region itself caused by the expansion or contraction of SSR or interruption (42).

In the present study, a total of 50 SSR markers which were dispersed throughout the 12 chromosomes, were used to assess the extent of genetic diversity across ten rice genotypes. RM1003, RM167, RM474, RM303, RM302, RM484, RM7117, RM271, RM525, RM6100, and RM236 were found to be polymorphic. Out of these markers, RM 6100 marker was specific for heat tolerance. This RM6100 marker is linked with a major quantitative trait locus (QTL) on chromosome 10 for heat stress tolerance at the flowering stage (43). Very recently, it was reported that induction of *DREB2A* gene expression is also associated with heat stress. It is reported as an important element of a transcriptional cascade in heat shock responses (44).

Polymorphism Information Content (PIC)

The Polymorphic information content (PIC) value calculated is shown in Table 4. 50 SSR primers were used across twenty-two rice accessions for the Polymorphic Information Content value detection. The PIC values for markers varied between 0 and 0.75. The primers which showed the highest PIC values were RM236 (0.75) followed by RM6100 (0.71).

In the present study, the PIC values among the SSR loci tested ranged 0.66 to 0.75, with an average of 0.67 per locus. Polymorphism information content (PIC) value reflects allele diversity and frequency among the genotypes. To measure the informativeness of each SSR marker, the PIC value was calculated. The PIC value is the indicator in predicting the usefulness of DNA markers for gene mapping, molecular breeding, and germplasm evaluation (45). Markers with higher PIC values possess the greater potential to reveal allelic variation. The average PIC value of SSR markers of different crops tested by different researchers varied based on the number of SSR markers used and the number of genotypes tested. The markers showed an average PIC value of 0.67 that almost showed higher polymorphism, which confirms that SSR markers used in this study were highly informative for genetic studies and extremely useful in distinguishing the polymorphic rate of a marker at a specific locus.

Genetic Diversity Analysis by Cluster Analysis

The genetic diversity among the genotypes was determined using the software program DendroUPGMA (46). The data were subjected to an unweighted pair groups method with arithmetic mean (UPGMA) analysis to generate a dendrogram (Fig. 33). The dendrogram indicated that the ten genotypes were clustered into two distinct clusters. Cluster 1 was the smallest, consisting of two genotypes, and cluster 2 contained two subclusters, cluster 2a and cluster 2b. The desirable heat-tolerant genotypes were identified and are scattered in

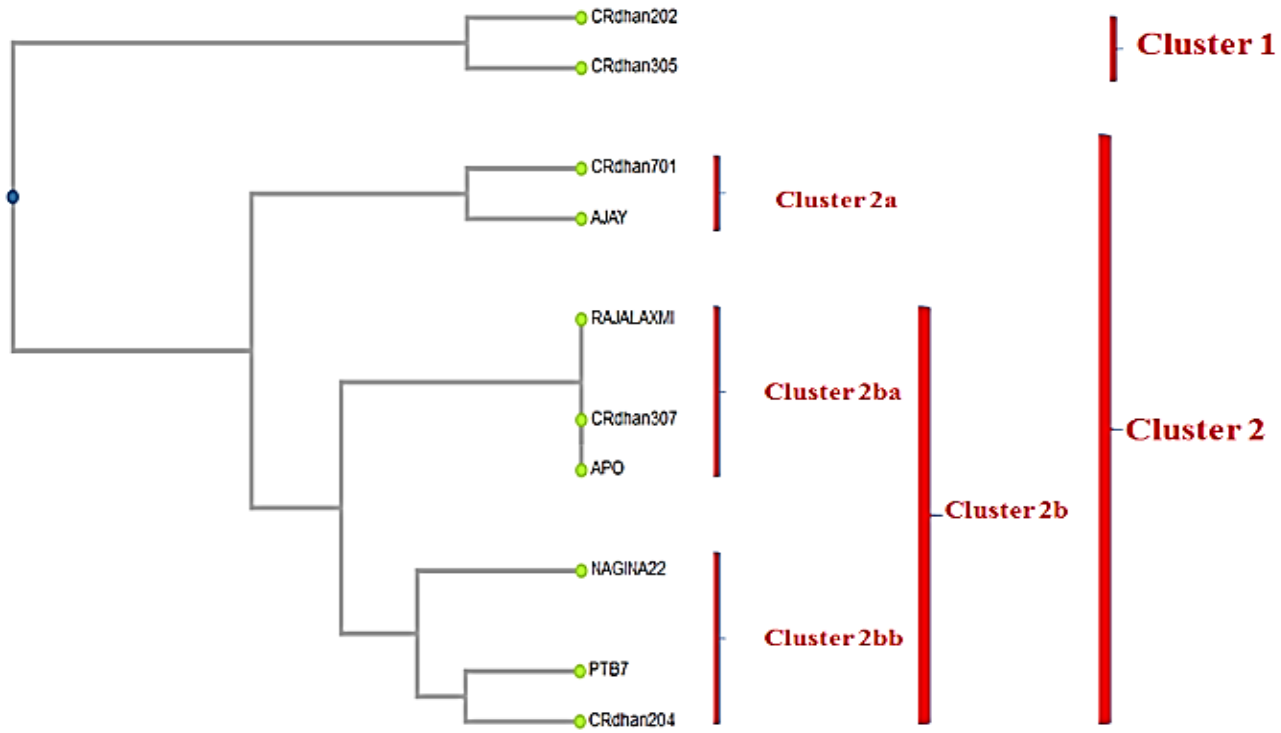


Figure 33. UPGMA based dendrogram for all the ten rice genotypes based on scoring data.

all clusters, but seven maximum desirable heat-tolerant genotypes are present in cluster 2.

The clustering of accessions was done using DendroUPGMA based on a similarity matrix using an unweighted pair group method with arithmetic mean (UPGMA) algorithm. The cluster analysis resolved the ten rice genotypes into two major clusters. A dendrogram was generated to analyse the relationships between the ten genotypes tested (Fig. 33). The genetic similarity index ranged from 0.1 to 0.833. The lowest value, 0.1 was obtained between PTB7 and CR-Dhan202, while the highest similarity value (0.833) calculated was between the PTB7 and CR-Dhan204 genotypes. Cluster 1 (CR-Dhan 202 and CR-Dhan 305) was the smallest with two genotypes. The desirable heat-tolerant genotypes were identified and are scattered in all clusters but maximum desirable genotypes are present in cluster 2.

Nagina 22, a selection from landrace Rajbhog in Nepal is a well-known drought and heat tolerant cultivar in Northern India. It has been used as a drought and heat tolerant donor for crop breeding for the last three decades in India. This study revealed two genotypes (Ptb- 7 and CR-Dhan 204) were closely similar to Nagina 22; these genotypes were drought-tolerant. Moreover, high temperature is closely related to drought stress in natural environments and often occurs in combination, and therefore, expression of the *DREB2A* gene can be induced by drought or heat shock alone or by a combination of drought and heat shock (47).

Conclusion

The variety N-22 was used a tolerant check and this is confirmed again in our study as it has significant difference over other genotypes regarding pollen viability and spikelet fertility, which are very critical parameters under stress. The variety CR-Dhan 307 with significantly higher mean pollen viability, spikelet fertility and 1000 grain weight can be utilized as a heat-tolerant variety. Other genotypes Ptb-7 and CR-Dhan 202 seemed to have tolerance traits beneficial at the vegetative stage. The genotype Rajalakshmi with its significantly reduced values in almost all parameters under heat stress, can be noted as a susceptible variety.

Identification of heat-tolerant genotypes is an essential requirement for developing heat-tolerant varieties. In this study, more diversity was observed between heat-tolerant and susceptible genotypes in SSR analysis. The marker RM6100 was found as a functional marker associated with heat tolerance in rice. The genetic diversity analysis with SSR markers will maximise the selection of diverse parents in the future rice breeding program or the development of heat-tolerant cultivars. Besides, it will help identify efficient strategies for the sustainable management of genetic resources of rice crops to cope with climate change. As the PIC value is an indication of the ability of the marker in indicating genetic diversity, the PIC values of the 11 polymorphic markers is useful for identify heat-tolerant genotypes. Through genetic diversity analysis using DendroUPGMA, the genotypes Ptb-7 and CR-Dhan 204 were found to be closely related to the heat-tolerant check variety, N-22 indicating genetically related traits for tolerance to heat.

The study is useful in identifying germplasm base in the future rice breeding program or for the development of the heat-tolerant cultivars. Besides, it will help in identifying efficient strategies for the sustainable management of the genetic resources of rice crops.

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Authors contributions

BR conceptualized the study and participated in its design and coordination. BR, SK, performed the phenotying work and taking observations. SK, NM, SS participated genotyping work. SK, SS assisted in data analysis. BR supervised and provided the resources for research study. All authors reviewed and approved the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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