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Molecular characterization and genetic variability analyses to identify heat tolerant gene of Maize in Pakistan

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Abstract
The overwhelming effect of global warming is an alarming concern of food security and economic growth in Pakistan. Effective solution is of supreme importance to genetically analyse maize germplasm. Mainly maize relies heavily on traditional breeding methods that take several years before the release of suitable maize varieties in Pakistan. In order to improve the maize germplasm, knowledge of available gene pool and variability among them needs to be ascertained as a precursor for other developmental breeding programs to boost up crop yield. Present research was conducted at two temperature regime (Islamabad and D.I.Khan) i.e. relatively cooler and warmer zones that will serve as antecedent for further research to be conducted around the belt Islamabad to D.I.Khan. Screening on the basis of morphological and biochemical analyses were performed and further confirmed by utilization of SSR markers. Thirty out of sixty genotypes showed heat tolerance at both locations confirmed through PCR which can be a source material for breeding purpose to develop heat tolerant hybrid production to fulfil the potential yield gap.

Keywords: heat tolerant gene, molecular characterization, genetic variance, climate change, biochemical analysis, SSR markers

Introduction
Among climate susceptibility and vulnerability Pakistan is ranked in top countries of the world Increase in extreme episodes of precipitation and temperature are affecting climate events and has adverse effect on economy mainly on agriculture in Pakistan. Climate change signifies a crucial and potentially irremediable danger to society and the planet [1]. Along with wheat and rice maize constitutes 30% calories to more than 4.5 billion people in ninety-four countries [2]. It is susceptible to severe climatic conditions and various other biotic and abiotic stresses. Maize (*Zea mays* L.) is one of the emerging crop which is mostly affected by adverse climatic conditions. Maize plants are prone to heat stress due to global warming [3, 4]. High temperature has deleterious impact on queen of cereal's productivity [5]. Heat stress is the main climatic factor that severely affect Maize crop production. Being C4 plant, it efficiently utilizes solar radiations and produces high yield with wide adaptation. However, crop production reduced under high temperature i.e. ≥35°C [6, 7]. Temperature near to 35°C at pollination and grain filling phase causes Maize production reduction up to 101 kg ha-1 per day. Heat stress causes a delay in tasseling and silking by increasing anthesis-silking interval which eventually reduce crop production due to poor fertilization and seed setting percentage. In South Asia maize yield is predicted to be reduced from 15–50% due to high temperature [8]. Genetic variability among morpho- physiological characters present in the cultivated germplasm along with the molecular characterization on heat tolerance basis in Pakistan will help in tracking down variety adoption. Due to minimum exploitation of genetic variation higher tolerance level against heat stress is not achieved yet [9]. Hence, there is much need to explore available gene pool against high temperature to select heat resistant genotypes. Therefore, present study was intended to explore genetic variability present in studied maize accessions for heat tolerance.

Materials and Methods
The present research was carried out in an open field of National Agricultural Research Centre, Islamabad (Pakistan) and Plant breeding and genetics Department, Gomal University, Dera Ismail Khan. Screening was done against sixty maize accessions (including one hybrid) named as 14965, 14985, 14986, 14990, 14991, 15056, 15061, 15065, 15066, 15067, 15080, 15085, 15100, 15105, 15115, 15116, 15121, 15125, 15131, 15170, 15175, 15185, 15190, 15205, 15206, 15005, 15015, 15035, 15036, 15055, 15261, 15266, 15267, 15281, 15306, 15326, 15336, 15341, 15346, 19174, 19175, 19176, 19177, 19178, 19179, 19180, 19181, 19182, 19183, 24669, 24670, 24674, 24679, 24680, 24684, 24695, 24699, 24700, 24771 and QPHM-200 for heat tolerance during spring season 2017. Twenty-one morpho-physiological parameters were taken to study and further confirmed by SSR markers. Accessions were grown in randomized complete block design with five replications. Row × row and plant × plant distance was kept 75 cm and 25 cm, respectively. All other recommended agronomic practices were performed. Morphological Characterization Data such as days to 50% germination, days to 50% tasseling, days to 50% silking, leaf length, leaf width, leaf area, no. of leaves, plant height, ear distance, ear length, ear girth, no. of kernels per ear, No. of kernels per row, 100 grain weight and grain yield were recorded at maturity stage. The recorded data were subjected to analysis of variance as given by [10]. The genotypic and phenotypic variances were calculated according to [11]. The genotypic and phenotypic coefficient of variances were estimated according to [12]. Broad sense heritability (H.B.S)

was estimated by as suggested by [13]. Genetic advance was measured according to formula given by [14].

Biochemical Analysis

Membrane stability index (MSI)

Membrane stability index was measured as proposed by Sairam (1994 [15]). Two sets of leaf samples were cut and placed in distilled water. One set kept at 40°C for 30 minutes and other at 100°C for 15 minutes. Electrical Conductivity (E.C1 and E.C2) measured with E.C meter:
MSI= (1- (E.C1/E.C2)) ×100

Relative Leaf water content (LRWC)

Relative water content was measured by applying [16] method, RWC = (F.W.-D.W.)/ (T.W.-D.W.) × 100 Where, F.W., T.W. and D.W. are fresh weight, turgid weight and dry weight (g), respectively.

Proline contents (PC)

Proline contents were measured as per protocol given by [17]. Plant material (0.1 g) was homogenized with 5ml 3% sulfo-salicylic acid, 5ml distilled water, glacial acetic acid and acid ninhydrin. Content in the tubes was heated in boiling water bath for one hour. Test tubes were then cooled and content was extracted with toluene (10ml) in a separating funnel. Toluene absorbance of toluene was recorded at 520nm. Proline umol/gm O.D e.g 0.7/standard value i.e 0.2916= 2.4 ((2.4*6)*4)/2=28.81 28.81/0.2 (fresh sample weight) .2916= standard proline value 6, 4, 2= dilution value, 6= total volume, 4= toluene, .2=sample size 2=filtrate

Pollen viability

Pollens were collected at 8 am and glass slides were prepared by using 1 % acetocarmine (1 drop). Prepared slides were observed under fluorescent microscope (lens 10 X).

Chlorophyll Content

Chlorophyll contents were measured using SPAD 502DL Plus at maturity stage.

Molecular Characterization of Heat Tolerant gene: Primer designing

Six heat tolerant gene specific markers was designed using NCBI primer- BLAST tool as shown in table 1. The primer was picked on the basis of high GC contents and low self-complementarity basis. PCR Analysis The PCR reagents optimization was done to check presence of heat tolerant genes (a. GRMZM2G148998 b. GRMZM2G115658 c. GRMZM2G537291 d. GRMZM2G324886 e. GRMZM2G436710 f. GRMZM2G094990) among genotypes under studied as 2µl of 10X Taq buffer, 2.5µl of 50 mM MgCl2, 0.4µl of 10 mM dNTPs, 10 mmol of forward and reverse primers in a final reaction volume, 0.3 unit of DNA Taq polymerase, 10.9 µl of DEPC-treated water and 2 µl of template DNA in order to make reaction volume up to 20 µl. Oligonucleotide primers were designed from already reported heat tolerant genes for maize in literature [18] as shown in table. Thirty- Seven cycles of amplification were run by using Applied Bioscience PCR System (96 wells) as per follows: denaturation at 94°C for 5 minutes, annealing at six different temperature with respect to primer for 45 seconds and extended at 72°C for 1 minute. Followed by amplification, 8 µl of PCR solution was electrophoresed on 2% Molecular grade agarose gel (Sigma) having ethidium bromide for 30 minutes at 100 Voltage in TAE buffer (40 mM Tris±acetate, 1 mM EDTA, pH 8.00) and then checked under Gel Documentation System. Data Analysis The data was analysed via Minitab 16 and R language for morphological characterization and molecular analysis and sequencing data was analysed using proper tools.

Table 1: Gene specific primers designed by using BLAST-NCBI tool

Gene Name	Chr. No	Qtls	Primer	Sequence (5'->3')	Length	Tm	Product Length
GRMZM2G148998	2	QPC1a, QHSI: FF, QHSI:MFa	F	CCGTACGACAACCAGAGGTG	20	60.39	129
			R	GATCTGGTGGAGCCGGATG	19	59.93	
GRMZM2G115658	2	QHSI:DYAa	F	ATGATGCCGTAGCAGACTAGC	21	60.00	671
			R	GATGAAGTTGGCGTAGGGGAT	21	59.86	
GRMZM2G537291	2	QHSI:DYAa	F	CGGCGCCCTGAATACG	16	57.78	311
			R	TCGATGTCCTTCCTGTTGCAC	21	60.61	
GRMZM2G324886	3	QHSI:DYb, QHSI:DYAb	F	CCTTGAGGGAGTTGAGCAAGA	21	59.65	301
			R	CAGGGAATCCAATATCAGAGAGGT	24	59.40	
GRMZM2G436710	5	QHSI:MFb, QPC1b	F	CTCACAGGGGCGATCTTCAA	20	59.75	241
			R	GTCTTGTCATGGCGGCTCTA	20	59.82	
GRMZM2G094990	9	QHSI:LS	F	TTTCCCAGATACGGTGCCTC	20	59.46	348
			R	CCCAGTACCCGGAGTTGTTC	20	60.04	

Results and Discussion

Analysis of variance depicted significant variances among studied accessions as shown in Table 2a, 2b, 2c. Genotypic and phenotypic coefficient of variances estimates ranged from 5.7-31.42 and 8.9-46.9, respectively which depicted close resemblance among all studied traits (Table 3). The GCV & PCV estimated highest relatedness with each other while ECV estimates were very less, depicted that the studied traits are genetically controlled showing very less impact of environment. Results were in close accordance to earlier findings of [19, 20]. Heritability was found higher for days to 50% germination, leaf length, stem diameter and grain yield which confirmed the genetic control for all studied characters. Higher broad sense heritability is a helpful tool to select best parental combination to produce hybrid with improved grain yield. Along with higher estimation of heritability and genetic advance was also higher for said attributes. Higher heritability and genetic advance might be due to additive genetic effects in all studied traits [21]. Grain yield is an effective tool to select better performing available germplasm. Grain yield comparison of Islamabad and D.I.Khan is showed through graphical presentation in Fig (a). These estimates assist plant breeders in effective genotype selection based on all studied attributes for future maize improvement program.

Correlation analysis

The correlation analysis measured the amount of association between two independent variables. Genotypic & phenotypic (rG & rP) coefficient of correlation were assessed for seed yield and contributing morpho-physiological traits as given in (Tables 4a, b and 5a, b). Genotypic and phenotypic correlation are deliberated as under:

Correlation between seed yield with other studied traits at Location 1

It was observed that seed yield exhibited a significant and positive genotypic and phenotypic correlation with days to 50% germination (DTG 50%), silking (DTS 50%), tasseling (DTT 50%), leaf length (LL), stem (SDia) and cob diameter (CDia), No. of leaves (NOL) and plant height (PH). Positive phenotypic correlation was observed for leaf (LL) and cob length (CL). It was also found that grain yield was genotypically and phenotypically non significantly correlated with leaf area as well as cob distance as shown in Table 4a, b. Results were found similar to [22-28].

Correlation between seed yield/Plant with other indicated traits at Location 2

It was observed that seed yield/plant showed a significant positive genotypicand phenotypic correlation with days to 50%germination (DTG 50%), silking, tasseling, leaf length, No. of leaves, cob distance, length and diameter. Grain yield was genotypically highly significant with No. of kernel/row. Non- significant genotypic and phenotypic correlation for grain yield was detected with leaf width, stem diameter, plant height and No. of kernel rows per cob as shown in table 5a,b. Significant correlation between grain yield with all characters designated the selection may be efficient to progress the maize seed yield. Results were quite similar to [22, 24, 25, 26, 27, 28, 29, 30].

Table 2a: Analysis of variance of morpho-physiological traits at both locations

Source	d.f	DTG 50%	DTT 50%	DTS 50%	LL	LW	LA	SG
Genotype	59	10.3*	49.3**	42.1**	372.9**	2.7*	3.76**	0.2**
Location	1	2.8	626.7**	1591.8**	24700.9**	2.3**	700584**	4.9**
Replication	2	1.2	17.3	7.7	4.5	0.5	2133	0.00536
Location*Genotype	59	5.3*	33.5**	16.6	163.1**	2.4**	16468**	0.2**
Error	238	1.1	11.6	13.9		1.3	6267	0.02**

Table 2b: Analysis of variance of morpho-physiological traits at both locations

Source	d.f	NOL	PH	EH	EL	EG	NOKRPE	NOKPR
Genotype	59	6.2**	1787.5**	1033.4**	18.863**	0.2**	9.2**	91.5**
Location	1	38.6**	98.2 N. S	108017.9**	14.601 N. S	0.5*	52.1**	134.4*
Replication	2	0.2*	885.2*	321.2*	48.401**	0.4*	11.2*	32*
Location*Genotype	59	5.1**	1309.8**	1558.9**	13.801N.S	0.3**	3.6 N. S	54.2**
Error	238	1.6	290	320.5	9.321	0.1	3.3	17.3**

Table 2c: Analysis of variance of morpho-physiological traits at both locations

Source	d.f	GY	GW 100	CC	MSI	LRWC	PC	PV
Genotype	59	1989.5**	107.5**	43984*	97.8**	150.7**	5.03**	142.5**
Location	1	12783.0 **	5.1*	50801*	1548.9**	616.2*	0.7*	2054.4**
Replication	2	21.5*	1.39*	39502 N. S	187.4*	27.2 N. S	0.3*	15.1*
Location*Genotype	59	378.4**	58.1**	44276*	107.04**	107.7**	3.3**	72.4**
Error	238	143.5**	17.6	44453	48.9	50.1	1.2	24.2**

** Highly significant, * significant, N.S Non significant

DTG50%=days to germination, DTT=Days to tasseling, DTS= Days to silking, LL=Leaf length, LW= Leaf width, LA=Leaf area, SG=Stem girth, NOL= No. of Leaves, PH= Plant height, EH= Ear height, EL= Ear length, EG= Ear girth, NOKRPE= No. of Kernel row per ear, NOKPR= No. of Kernels per row, GY=Grain yield, GW=Grain weight, CC=Chlorophyll Contents, MSI=Membrane Stability Index, LRWC= Leaf relative water content, PC= Proline content, PV=Pollen viability

Table 3: Genetic parameters of studied traits at Islamabad and D.I.Khan

Traits	Mean	MST	MSE	Vg	Ve	Vp	GCV%	ECV%	PCV%	Hbs%	GA%Mean
DTG	6.7	10.3	1.1	3.1	1.1	4.2	26.0	15.6	30.3	73.5	46.0
DTT	48.6	49.3	11.6	12.6	11.6	24.2	7.3	7.0	10.1	52.0	10.8
DTS	54.0	42.1	13.9	9.4	13.9	23.3	5.7	6.9	8.9	40.3	7.4
LL	77.2	372.9	31.0	114.0	31.0	145.0	13.8	7.2	15.6	78.6	25.3
LW	7.8	2.7	1.3	0.4	1.3	1.8	8.6	14.9	17.2	25.1	8.9
LA	448.4	23570.0	6267.0	5767.7	6267.0	12034.7	16.9	17.7	24.5	47.9	24.2
SDIA	0.8	0.2	0.0	0.1	0.0	0.1	31.4	16.0	35.3	79.5	57.8
NOL	11.3	6.2	1.6	1.5	1.6	3.1	10.9	11.2	15.6	48.6	15.6
PH	145.4	1787.5	290.0	499.2	290.0	789.2	15.4	11.7	19.3	63.3	25.2
CD	99.7	1033.0	321.0	237.3	321.0	558.3	15.5	18.0	23.7	42.5	20.8

CL	16.8	18.9	9.3	3.2	9.3	12.5	10.6	18.2	21.0	25.4	11.0
CDIA	1.3	0.2	0.1	0.1	0.1	0.2	16.3	24.8	29.7	30.4	18.6
NOKRPC	12.6	9.3	3.3	2.0	3.3	5.3	11.2	14.5	18.3	37.3	14.1
NOKPR	21.3	91.5	17.3	24.7	17.3	42.0	23.4	19.6	30.5	58.9	37.0
GY	89.1	1989.5	143.5	615.3	143.5	758.8	27.9	13.5	30.9	81.1	51.8
SW	23.6	107.5	17.6	30.0	17.6	47.6	23.2	17.8	29.2	63.0	38.0
CC	41.6	48.9	16.1	10.9	16.1	27.0	7.9	9.6	12.5	40.4	10.4
MSI	39.0	97.8	48.9	16.3	48.9	65.2	10.4	17.9	20.7	25.0	10.7
LRWC	45.7	150.7	50.1	33.6	50.1	83.6	12.7	15.5	20.0	40.1	16.6
PC	3.6	5.0	1.7	1.1	1.7	2.8	29.6	36.3	46.9	40.0	38.7
PV	69.7	142.5	24.2	39.5	24.2	63.6	9.0	7.1	11.4	62.0	14.6

MST= treatment mean square, Vg=Genotypic variance, Ve= Environmental variance, Vp= Phenotypic variance, GCV%= Genotypic coefficient of variance, ECV= Environmental coefficient of variance, PCV= Phenotypic coefficient of variance, Hbs= Broad sense heritability, GA= Genetic advance, DTG50%=days to germination, DTT=Days to tasseling, DTS= Days to silking, LL=Leaf length, LW= Leaf width, LA=Leaf area, SG=Stem girth, NOL= No. of Leaves, PH= Plant height, EH= Ear height, EL= Ear length, EG= Ear girth, NOKRPE= No. of Kernel row per ear, NOKPR= No. of Kernels per row, GY=Grain yield, GW=Grain weight, CC=Chlorophyll Contents, MSI=Membrane Stability Index, LRWC= Leaf relative water content, PC= Proline content, PV=Pollen viability

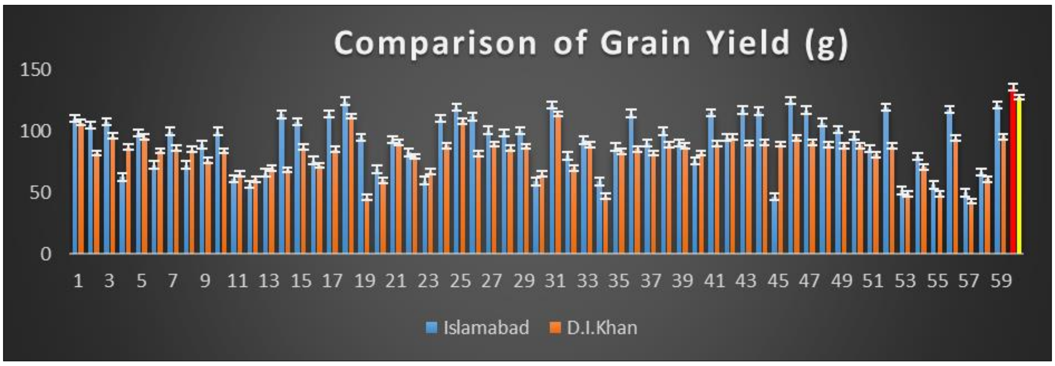


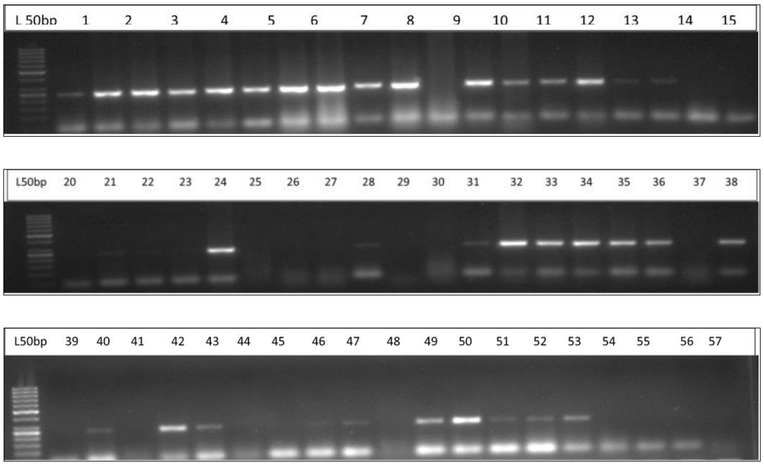
Fig 1a: Graphical presentation of grain yield at both locations

Molecular analysis

Heat tolerant six primers were designed and picked on the basis of GC content percentage. These primers were applied on all 60 maize varieties. Out of six primers only one marker against GRMZM2G436710 was optimized others did not anneal at any of the temperature. This marker was further used to characterize 60 varieties with respect to heat tolerant gene.

PCR Results

Optimization for the six primers was carried out at different temperature ranged from 48-64°C against these six genes (a. GRMZM2G148998 b. GRMZM2G115658 c. GRMZM2G537291 d. GRMZM2G324886 e. GRMZM2G436710 f. GRMZM2G094990). Only primer for this heat tolerant gene i.e. GRMZM2G436710 gave the sharp bands during optimization (Fig b). This primer was further used to characterize the 60 maize varieties against heat tolerant gene. Out of 60 maize varieties 30 varieties give sharp band of 241 bp in size on 1.8 % agarose gel run at 80V for 45 minutes. The PCR results were confirmed thrice.



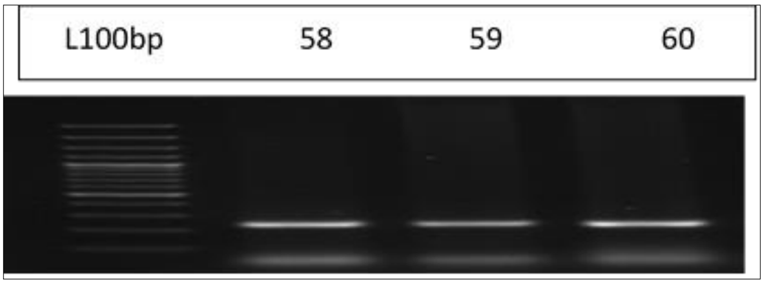


Fig 2b: Heat tolerant germplasm identification through PCR banding pattern

Conclusion and Recommendation

To the best of our knowledge we are reporting the heat tolerant gene presence in the maize genotypes i.e. 15190, 15056, 15206, 15336, 24669, 15341, 14990 and QPHM 200 in Pakistan. Morpho-physiological characterization also support to yield best at two temperature regime. Presence of heat tolerant gene is confirmed through PCR. However, further study is recommended for full length heat tolerant gene analysis in future for studied lines.

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