Unraveling multi-trait genetic control of drought tolerance and yield stability in black gram via genome-wide association analysis

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Abstract

Drought stress is a major abiotic constraint limiting productivity of black gram (*Vigna mungo* L.) in rainfed agro-ecosystems, necessitating the identification of drought-resilient genotypes and associated adaptive traits. In the present study, a genome-wide association approach integrating morpho-physiological, biochemical and molecular analyses was employed to unravel the genetic architecture of drought tolerance, grain yield and its component traits. A panel of 60 diverse genotypes were evaluated under control and water-stress conditions using a randomized complete block design. Significant genotypic, treatment and genotypic × treatment variances were observed for most traits, indicating substantial genetic variability and differential drought responses. Drought stress caused marked reductions in seed yield, plant height, chlorophyll content, relative water content, membrane stability index, NDVI and canopy temperature depression, while proline and total soluble sugar contents increased substantially, reflecting osmotic adjustment mechanisms. Among genotypes IC546466, IPU9612, IPU243, IC331232 and IC426766 exhibited superior yield stability and physiological resilience under stress. Genotyping with 130 SSR markers revealed high allelic diversity (389 alleles; mean PIC = 0.57) and clear population stratification into two subpopulations. Genome-wide association analysis identified 28 and 40 significant marker-trait associations under control and drought stress conditions respectively, highlighting their potential utility in marker-assisted selection. Overall, these associations underscore the critical role of intrinsic tolerance mechanisms and cumulative traits in drought tolerance, identifying promising genotypes and SSR markers to accelerate breeding programs for drought-resilient crops.

Introduction

Crop phenology and crop yield are strongly influenced by the prevailing weather conditions throughout the growing season, irrespective of geographical location (Akter and Islam 2017). Rising atmospheric temperatures and increasing water scarcity are major constraints affecting growth and development of pulse crops in several states of India (Venugopalan et al. 2021). While global food security in the 21st century is under serious threat due to the escalating frequency of heat and drought stress caused by climate change, particularly across tropical and subtropical regions (Mahilane et al. 2017). Among abiotic stresses, drought remains the most detrimental factor contributing to yield reductions (Vinocur and Altman 2005). It hampers the crop's capacity to complete its life cycle, with prolonged dry spells during key different phenological stages. Under such conditions, plants need to adapt through a range of morpho-physiological and biochemical responses to mitigate the effects of water deficit. However, the severity and duration of stress, along with the crop's phenological stage, strongly influence its impact on growth and productivity (Sarkar et al. 2025).

In most germplasm screening studies for drought tolerance, seed yield is considered the primary criterion. However, for effective breeding programs, identification of secondary traits contributing to drought resilience is equally essential. Drought stress adversely affects morphological, physiological and biochemical processes that culminate in poor grain yield (Baroowa and Gogoi 2012, 2013; Baroowa et al.

2016; Maheswari et al. 2016). Higher intensity and longer duration of stress accelerate chlorophyll degradation (Kiani et al. 2008) and inhibit photosynthetic activity by damaging the photosynthetic apparatus (Manivannan et al. 2007). Nearly all development processes are affected by drought, though several morphological and physiological traits conferring drought tolerance have been reported in crop species. Hence, identifying and characterizing these adaptive traits within existing germplasm resources is imperative for breeding drought-tolerant cultivars.

Black gram (*Vigna mungo* L.), a short-duration pulse crop belonging to the family *Fabaceae*, is predominantly cultivated under rainfed conditions in tropical and subtropical regions of South Asia (Sarkar et al. 2026). It is an important grain legume for its rich protein content and its role in maintaining soil fertility through biological nitrogen fixation (Baroowa and Gogoi 2016). Despite its economic and nutritional significance, black gram productivity remains very low and affected by abiotic stresses, particularly drought (Vanaja et al. 2006). The crop exhibits both photo- and thermo-sensitivity and its indeterminate flowering habit leads to simultaneous vegetative and reproductive growth, creating competition for assimilates and resulting in a low harvest index with reduced grain yield (Somta et al. 2019; Sahu et al. 2020). Moreover, the expansion of black gram cultivation is restricted by the lack of genetic, genomic resources and limited genetic diversity (Chaitieng et al. 2006; Gupta et al. 2008; Somta et al. 2019).

A systematic approach encompassing the genetic and genomic characterization and utilization of diverse germplasm is essential for unraveling the genetic architecture of key agronomic traits. Genome-wide association studies (GWAS) serve as an effective strategy for dissecting complex traits by exploiting natural allelic variation within populations. GWAS has been extensively applied in legume crops such as soybean (Hwang et al. 2014), chickpea (Plekhanova et al. 2017), cowpea (Xu et al. 2017), pigeonpea (Varshney et al. 2009) and mungbean (Sokolkova et al. 2020), providing valuable insights into the genetic basis of productivity and stress adaptation (Lorenz et al. 2010; Scherer and Christensen 2016).

In the present study, genome-wide association analysis was conducted on a diverse set of black gram germplasm to assess genetic diversity, population structure and identify marker trait associations (MTAs) related to yield and its component traits. The outcomes of this research are a crucial genomic resource to facilitate the genetic dissection and improvement of productivity-related traits in black gram.

Materials and Methods

Plant Materials

An initial evaluation of 375 germplasm accessions, including advanced breeding lines was conducted for yield and yield-related traits under rainfed conditions (data not presented). From this set, sixty black gram genotypes with superior performance and representing diverse agro-ecological origins were selected for the present study (Supplementary Table 1). These genotypes encompassed varieties (PDU1,

WBG108, IPU941, LBG787, IPU243) released for different regions and advanced breeding lines obtained from research centres operating under All India Coordinated Research Program (AICRP) on Black gram, Indian Council of Agricultural Research (ICAR). The selected materials represent a wide geographical distribution across the major black gram growing regions of India.

Experimental Design

The field experiments were conducted at the ICAR-Central Research Institute for Dryland Agriculture (CRIDA), Hayathnagar Research Farm (17°27'N latitude, 78°35'E longitude, 515 m above mean sea level) located on the Deccan Plateau in southern India, during the *Kharif* season of 2023. The trails were laid out in a randomized complete block design (RCBD) with three replications under two moisture regimes, control (well-watered) and water-stress (drought) conditions. Each genotype was grown in a plot measuring 5.4 m^2, consisting of two rows of 3 m each, with 30 cm row spacing and 10 cm plant spacing. Fertilizers were applied as per the recommended package of practices (20:40:20kg N:P:K ha^{-1}). In the stress treatment, irrigation was withheld from the flowering stage until visible drought symptoms appeared. Soil moisture was monitored using a TDR moisture sensor from flowering to pod-setting stages (36–78 days after sowing). The soil moisture content of control experiment varied between 35–40% while under stress treatment it varied between 10–12%.

Phenotypic Evaluation

Observations were recorded on three representative plants from each plot for plant height (PH), number of branched (BN), number of clusters (CN), pod length (PL), number of seeds per pod (SPP), hundred seed weight (HSW) and seed yield per plant (SY). Days to 50% flowering (DF) were recorded on a plot basis under both moisture regimes for two consecutive years.

Physiological and Biochemical Observations

Physiological traits were recorded under both control and water-stress treatments. Chlorophyll content was estimated using a SPAD meter (Soil Plant Analysis Development) on the fully expanded third leaf from the top, between 10:00 and 11:00 AM. The Normalized Difference Vegetation Index (NDVI) was recorded to assess canopy greenness and photosynthetic activity. Canopy temperature was measured using a handheld infrared thermometer following the method of Yousif et al. (2019), targeting the leaf canopy at a 45° angle under clear, calm afternoon conditions (2:00–3:00 p.m.). Canopy temperature depression (CTD) was calculated as the difference between air and canopy temperatures.

Relative Water Content (RWC)

Fully expanded young leaves were sampled to measure RWC following Barrs and Weatherley (1962). The fresh weight (FW) was recorded, followed by soaking in distilled water for 4 hours to obtain the turgid weight (TW). Samples were over-dried at 70°C to determine the dry weight (DW). RWC was calculated as:

$$\text{RWC (\%)} = (FW-DW)/(TW-DW) \times 100$$

Chlorophyll Estimation (mg g^{-1} FW)

Chlorophyll was extracted using the dimethyl sulfoxide (DMSO) method (Barnes et al. 1992). Around 0.1 g of fresh leaf tissue was incubated in 10 mL of DMSO overnight. Concentrations of chlorophyll a, b and total chlorophyll were determined at 663 and 645 nm absorbance using specific coefficients, expressed in mg chlorophyll g^{-1} fresh weight.

Leaf Proline Content (µg g^{-1} FW)

Proline accumulation, indicative of osmotic adjustment was quantified following Bates et al. (1973). Fresh leaf tissue (0.5g) was homogenized in 3% sulfosalicylic acid and reacted with acid ninhydrin. Absorbance of the toluene extract was read at 520nm and proline concentration was determined from standard curve.

Total Soluble Sugar Content (mg g^{-1} FW)

Soluble sugars were determined by the phenol-sulfuric acid method as described by Kochert (1978). Leaf material (100 mg) was extracted in 70% ethanol, centrifuged and reacted with phenol and concentrated sulfuric acid. After color development, absorbance was measured at 490 nm and sugar concentration was calculated.

Membrane Stability Index (MSI)

Membrane stability was measured based on electrolyte leakage following Deshmukh et al. (1991). Uniform leaf discs (100 mg) were placed in distilled water and divided into two sets, one kept at room temperature (C_1) and another heated at 100°C for 15 min (C_2). MSI was computed using:

$$MSI = [1- (C_1/C_2)] \times 100$$

Genotyping

Genomic DNA was extracted from young leaves (2–3 weeks old) using the CTAB method (Doyle and Doyle, 1987). A total of 130 SSR markers, including 58 genomic (Jegadeesan et al 2015) and 72 genic (Souframanien et al. 2015) markers (Supplementary Table 2a, 2b) were utilized for generating genotyping data. PCR amplification was carried out in a 20 µL reaction containing 50 ng genomic DNA, 1 µL each of forward and reverse primers (10 pmol) and 10 µL of 2 × EmeraldAmp GT PCR Master Mix

(Takara). The cycling conditions included an initial denaturation at 95°C for 5 min, 35 cycles of denaturation, annealing at 55–60°C for 45sec and extension at 72°C for 45 sec, followed by a final extension at 75°C for 5 min. Amplified products were separated on 3% agarose gels containing ethidium bromide, using 1×TBE buffer at 70V for 1.5 h and visualized in a gel documentation system (BIO-RAD). Allele sizes were determined relative to a 50 bp DNA ladder and were scored for diversity analysis using the Bio-Rad Image Lab software.

Phylogenetic analysis and population structure

Cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) was performed in PowerMarker v3.25 (Nei 1983). Allele number, gene diversity, heterozygosity, inbreeding coefficient and polymorphic information content (PIC) were calculated for each SSR locus (Liu and Muse 2005). Population structure was inferred using STRUCTURE v2.3.4 (Pritchard et al. 2000) based on polymorphic SSRs. Five independent runs were executed for each assumed number for populations (K = 1–10) with a burn-in period and MCMC iteration of 1,00,000 each. The optimal number of subpopulations (K) was inferred using the ΔK method (Evanno et al. 2005). Among the runs, K = 2 showing the highest likelihood was selected to assign the posterior membership proportions and was subsequently used as a covariate in the TASSEL v2.0.1 program (Bradbury et al. 2007) for marker-trait association analysis. Association between SSR markers and morphological or physiological traits ($p < 0.05$) were evaluated using the General Linear Model (GLM).

Statistical Analysis

Phenotypic data were analysed using SAS software (Version 9.3). Analysis of variance (ANOVA) and least significant difference (LSD) tests were conducted to determine significant genotypic differences under individual and combined water regimes.

Results

Analysis of variance (ANOVA) demonstrated significant variances ($p < 0.01$) for treatment, genotype and their interactions for most traits (Supplementary Table 3). The significant genotypic variances across traits indicated substantial genetic diversity among genotypes. Except for days to flowering (DF), branch number (BN), seeds per pod (SPP) and hundred seed weight (HSW), all traits also exhibited significant genotype × treatment (G × T) interactions. The higher mean values were observed for most of the morphological, physiological and yield-related traits under control to those under drought stress conditions. Conversely, total soluble sugars (TSS) and proline contents increased significantly under drought conditions, reflecting their potential roles in osmotic adjustment and stress adaptation. Furthermore, genotype-specific responses varied considerably across treatments, emphasizing differential adaptive potential among genotypes with IC546466, IPU9612, IPU243, IC426766 and IC331232 performed better under both conditions.

Morphological and physiological responses

The experiment revealed significant differences among black gram genotypes under control and stress conditions across all quantitative traits. Phenological and morphological attributes were notably affected by stress treatments. Mean days to flowering (DF) declined from 37.02 days under control to 35.36 days under stress, indicating an accelerated phenological changes. Likewise, plant height (PH) declined from 33.7 cm to 28.31 cm and reductions were also evident in branch (BN) and cluster number (CN), suggesting restricted vegetative and reproductive growth under drought stress conditions. The average seed yield (SY) declined sharply from 8.25 g plant^{-1} (control) to 5.85 g plant^{-1} (stress), representing an approximate 29.09% yield loss relative to control. Corresponding declines were also observed for pod length (PL) and hundred seed weight (HSW), collectively indicating reduced productivity under water-limited conditions. Physiological indices such as relative water content (RWC) and membrane stability index (MSI) also substantially reduced under stress, signifying tissue dehydration and cellular membrane injury.

Drought stress also caused discernible declines in total chlorophyll content (4.15 to 3.03 mg g^{-1} FW) and SPAD readings (43.75 to 40.26). Conversely, stress-responsive metabolite increased by approximately 146% for proline and 78.8% for total soluble sugars (TSS) over control, denoting osmotic regulation under stress (Supplementary Table 4). The normalized difference vegetation index (NDVI) and canopy temperature depression (CTD) declined significantly, consistent with reduced photosynthetic efficiency and diminished canopy cooling. The mean performance of the genotypes was evaluated for both control and stress conditions, among them IC436606, IC401376, IC257676, IPU243 and IC546466 exhibited superior yield (Supplementary Table 5). IPU9612 and IPU941 recorded higher SPAD and CTD values respectively, reflecting enhanced photosynthetic tolerance. Conversely, IC436635 and IC436621 experienced severe yield reductions under drought. The overall coefficient of variation (CV%) ranged from 1.86% (DF) to 16.42% (BN), reflecting moderate variability among black gram genotypes for different traits, whereas significant genotype × treatment interactions for most traits when compared between interaction effects.

Molecular genetic diversity using SSR markers

All 60 genotypes were genotyped using 130 SSR markers of which 58 were genomic and 72 were genic linked with various drought-responsive traits. Among these, one marker (VmGEN9) was found to be monomorphic and rest polymorphic. These polymorphic SSRs amplified a total of 389 alleles with a mean of 3 alleles per locus (Supplementary Table 6) with a total of 143 unique alleles. The highest allelic count (7 alleles) was observed in VmgEST79, followed by VmGEN4, VmEST35, VmEST48 and VmgEST61 (each with 6 alleles). The remaining markers generated in between 2–5 alleles, underscoring a considerable degree of genetic variability among genotypes. While allele sizes ranged from 105 to 807 bp with VmEST34, VmgEST33 and VmgEST79 amplifying larger fragments and VmgEST81 producing the smallest (105 bp). The polymorphic information content (PIC) ranged from 0.00 (VmGEN9 - monomorphic SSR) to 0.79 (VmgEST61) averaging 0.57, while gene diversity values varied from 0.00 to 0.82. SSR marker profile produced by the primer pair VmgEST79 and VmgEST61 was shown in **Fig. 1a and 1b**. These marker data used for cluster analysis following UPGMA method (Nei, 1983), revealed two

major clusters each further divided into four sub-clusters - Cluster I (14 genotypes), Cluster II and III (15 genotypes each) and Cluster IV (16 genotypes) (**Fig. 2**). This grouping pattern reflects the considerable diversity within the studied population. The population structure analysis corroborated these results, assigning the genotypes into two subpopulations (P1 = 29 and P2 = 31) based on the Evanno ΔK method (K = 2) (**Figs. 3 & 4**), providing finer resolution of genetic grouping and potential introgression events among accessions.

Marker-trait association analysis

The GLM model (Q matrix-based) detected significant markers-trait associations ($p < 0.05$, $R^2 > 0.15$) across control and stress conditions (Tables 1 and 2). Under control conditions, 28 significant associations were identified for 11 morpho-physiological traits. For instance, CTD was associated with VmEST26, VmGEN29, VmgEST72, VmgEST79 and VmgEST10 while MSI was linked with VmgEST2, VmGEN4 and VmEST48. NDVI correlated with VmEST25 and VmgEST23, RWC and SPAD with VmgEST72 and VmgEST77. Among agronomic traits, plant height was associated with VmgEST61 and cluster number with eight markers (VmGEN21, VmgEST25, VmgEST75, VmEST11, VmgEST72, VmgEST61, VmgEST10, VmEST2). HSW with VmgEST25, VmGEN19 and VmEST5, while pod length with VmgEST82 and VmgEST16. Seed yield and DF with VmGEN19 and VmGEN41 respectively.

Table 1
Association between SSR markers and morpho-physiological traits evaluated under control condition

Trait	Locus	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
CN	VmGEN21	10.14	0.002	0.15	0.15
CN	VmgEST25	3.3	0.03	0.16	0.16
CN	VmgEST75	3.82	0.01	0.17	0.17
CN	VmEST11	4.04	0.01	0.18	0.18
CN	VmgEST72	4.1	0.01	0.18	0.18
CN	VmgEST61	2.69	0.03	0.20	0.20
CN	VmgEST10	4.78	0.005	0.21	0.21
CN	VmEST2	8.9	4.40E-04	0.24	0.24
CTD	VmEST26	2.87	0.03	0.18	0.17
CTD	VmGEN29	5.96	0.005	0.18	0.17
CTD	VmgEST72	4.9	0.004	0.21	0.21
CTD	VmgEST79	2.69	0.02	0.24	0.24
CTD	VmgEST10	6.56	7.17E-04	0.27	0.26
DF	VmGEN41	10.84	0.0017	0.21	0.15
HSW	VmgEST25	3.34	0.03	0.20	0.15
HSW	VmGEN19	6.57	0.0027	0.22	0.18
HSW	VmEST5	6.76	0.0023	0.23	0.19
MSI	VmgEST2	3.9	0.01	0.30	0.15
MSI	VmGEN4	2.7	0.03	0.33	0.17
MSI	VmEST48	3.42	0.01	0.36	0.21
NDVI	VmEST25	5.96	0.0045	0.22	0.17
NDVI	VmgEST23	8.19	7.61E-04	0.27	0.21
PH	VmgEST61	3.22	0.01	0.46	0.17
PL	VmgEST82	3.25	0.03	0.17	0.15

Where, BN= branch number, Chlo= total chlorophyll content (mg/gm FW), CN= cluster number, CTD= Canopy Temperature Depression (°C), DF= days to 50% flowering, HSW= hundred seed weight, MSI= membrane stability index (%), NDVI= normalized difference vegetation index, PH= Plant height (cm), PL = pod length (cm), RWC= relative water content (%), SPAD= chlorophyll meter reading (SCMR), SPP= seeds per pod, SY= seed yield (g/plant).

Trait	Locus	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
PL	VmgEST16	5.1	0.01	0.18	0.15
RWC	VmgEST72	4.36	0.01	0.22	0.18
SPAD	VmgEST77	9.51	2.78E-04	0.26	0.25
SY	VmGEN19	4.79	0.01	0.15	0.15

Where, BN= branch number, Chlo= total chlorophyll content (mg/gm FW), CN= cluster number, CTD= Canopy Temperature Depression (°C), DF= days to 50% flowering, HSW= hundred seed weight, MSI= membrane stability index (%), NDVI= normalized difference vegetation index, PH= Plant height (cm), PL = pod length (cm), RWC= relative water content (%), SPAD= chlorophyll meter reading (SCMR), SPP= seeds per pod, SY= seed yield (g/plant).

Table 2
Association between SSR markers and morpho-physiological traits evaluated under stress condition

Trait	Locus	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
BN	VmEST40	3.16	0.03	0.15	0.15
CN	VmEST9	5.02	0.01	0.19	0.15
CN	VmEST31	3.67	0.02	0.20	0.16
CN	VmgEST75	3.78	0.02	0.21	0.16
CN	VmgEST10	3.85	0.01	0.21	0.17
CN	VmGEN29	7.19	0.0043	0.24	0.20
CN	VmEST28	5.23	0.0001	0.26	0.21
CN	VmEST1	6.1	0.00047	0.39	0.37
CTD	VmGEN4	2.71	0.03	0.26	0.19
HSW	VmgEST80	3.51	0.02	0.22	0.15
HSW	VmGEN68	5.84	0.01	0.23	0.16
HSW	VmEST5	10.71	0.00023	0.32	0.26
MSI	VmgEST2	4.5	0.01	0.34	0.16
NDVI	VmgEST2	3.26	0.03	0.15	0.15
NDVI	VmEST20	3.49	0.02	0.16	0.16
PH	VmgEST61	3.07	0.02	0.33	0.20
PH	VmEST19	9.47	0.001	0.35	0.22
RWC	VmGEN58	4.07	0.01	0.33	0.15
RWC	VmgEST75	4.12	0.01	0.34	0.15
RWC	VmEST28	4.17	0.01	0.34	0.15
RWC	VmGEN39	13.67	4.02E-04	0.34	0.16
RWC	VmGEN31	7.24	0.007	0.35	0.17
RWC	VmgEST61	3.52	0.01	0.39	0.20
SPAD	VmEST49	3.11	0.03	0.17	0.15

Where, HSW= hundred seed weight, MSI= membrane stability index (%), NDVI= normalized difference vegetation index, PH= Plant height (cm), PL = pod length (cm), RWC= relative water content (%), SPAD= chlorophyll meter reading (SCMR), SPP= seeds per pod, SY= seed yield (g/plant), TSS= total soluble sugar (mg/g FW).

Trait	Locus	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
SPAD	VmgEST80	3.57	0.02	0.17	0.16
SPAD	VmEST5	6.18	0.001	0.19	0.18
SPAD	VmGEN33	12.94	0.0001	0.19	0.18
SPAD	VmgEST77	6.56	0.005	0.20	0.19
SPAD	VmGEN54	4.54	0.01	0.21	0.20
SPAD	VmgEST58	7.93	6.84E-04	0.23	0.22
SPAD	VmgEST82	5.92	0.0007	0.25	0.24
SPP	VmgEST69	5.01	0.01	0.18	0.15
SPP	VmEST49	2.96	0.04	0.16	0.15
SPP	VmGEN35	9.6	0.0091	0.28	0.25
SPP	VmGEN37	22.41	0.00001	0.30	0.27
SY	VmEST36	3.54	0.02	0.18	0.16
TSS	VmEST27	4.32	0.01	0.19	0.19

Where, HSW= hundred seed weight, MSI= membrane stability index (%), NDVI= normalized difference vegetation index, PH= Plant height (cm), PL = pod length (cm), RWC= relative water content (%), SPAD= chlorophyll meter reading (SCMR), SPP= seeds per pod, SY= seed yield (g/plant), TSS= total soluble sugar (mg/g FW).

Under drought stress, 37 marker trait associations were detected across 12 traits. CTD was associated with VmGEN4; MSI with VmgEST2; NDVI with VmgEST2 and VmEST20; and RWC with six markers (VmGEN58, VmgEST75, VmEST28, VmgEST39, VmGEN31, VmgEST61). SPAD showed strong associations with eight markers (VmEST49, VmgEST80, VmEST5, VmGEN33, VmgEST77, VmGEN54, VmgEST58, VmgEST82), while TSS associated with VmEST27. For agronomic traits, PH was linked with VmgEST61 and VmEST19; BN with VmEST40 and cluster number with VmEST9, VmEST31, VmgEST75, VmgEST10, VmGEN29, VmEST28 and VmEST1. HSW was related to VmgEST80, VmGEN68 and VmEST5; Seed yield with VmEST36, whereas SPP to VmgEST69, VmEST49, VmGEN35 and VmGEN37. Consistent associations across conditions were detected for five marker-trait pairs spanning four traits (Table 3). VmgEST75 and VmgEST10 with CN, VmEST5 with HSW, VmgEST2 with MSI and VmgEST77 with SPAD.

Table 3
Common SSR markers with significant association for morpho-physiological traits under stress and control condition

Trait	Treatment	Locus	F_Marker	p_Marker	Rsq_Model	Rsq_Marker
CN	Stress	VmgEST75	3.78	0.02	0.21	0.16
CN	Control	VmgEST75	3.82	0.01	0.17	0.17
CN	Stress	VmgEST10	3.85	0.01	0.21	0.17
CN	Control	VmgEST10	4.78	0.005	0.21	0.21
HSW	Stress	VmEST5	10.71	0.00023	0.32	0.26
HSW	Control	VmEST5	6.76	0.0023	0.23	0.19
MSI	Stress	VmgEST2	4.5	0.01	0.34	0.16
MSI	Control	VmgEST2	3.9	0.01	0.3	0.15
SPAD	Control	VmgEST77	9.51	2.78E-04	0.26	0.25
SPAD	Stress	VmgEST77	6.56	0.005	0.20	0.19

Where, CN= cluster number, HSW= hundred seed weight, MSI= membrane stability index (%), SPAD= chlorophyll meter reading (SCMR).

Discussion

Broadening the genetic base of cultivars through introgression of novel alleles from closely related or more distant species remains a cornerstone of modern crop improvement, particularly for complex stresses such as drought (Raja et al. 2022; Kachave et al. 2015). Systematic evaluation of genetic diversity in germplasm collections is therefore essential to identify unique, genetically divergent genotypes that can serve either as elite donors in hybridization programmes or as a potential variety. Traditionally, morphological and agronomic descriptors have been widely used to quantify diversity and trait associations. However, their expression is often confounded by environmental influences, limiting their reliability when used alone. In this context, an integrated framework combining agro-morphological, physiological and molecular marker information provides a more robust and comprehensive understanding of diversity patterns and enables effective expansion of the genetic base for key adaptive traits. Guided by this rationale, the present study evaluated black gram genotypes using both field-based traits and SSR markers to identify promising donors for future improvement programmes.

The utility of physiological traits in crop improvement depends on their strength of association with yield, magnitude of genetic variation and the nature of genotype × treatment interactions (Gurumurthy et al. 2019). In this investigation, significant variation among black gram genotypes across seasons for multiple morpho-physiological traits indicated the presence of adequate exploitable diversity for breeding. The observed differences in traits such as chlorophyll content, SPAD values, RWC, MSI, proline,

TSS, NDVI and CTD under contrasting moisture regimes underscore their potential as secondary selection criteria for drought tolerance. Importantly, understanding the heritability of these traits under both stress and non-stress conditions, as well as their inter-trait correlations, is critical for predicting selection response and designing efficient selection indices.

Under drought stress, decline in plant height, branch and cluster numbers, pods per plant and hundred seed weight demonstrated a suppression of vegetative and reproductive growth process due to moisture limitation (Kumar et al. 2020). Such reductions are frequently associated with reduced carbon assimilation and internal resource allocation under stress (Baroowa and Gogoi 2012). The accelerated flowering observed under stress reflects an adaptive escape mechanism, enabling plants to complete their life cycle before severe soil water deficit intensifies (Cortes and Suidaria 1986; Uprety and Bhatia 1989).

Significant reductions in chlorophyll content, SPAD values, RWC and MSI indicate deleterious effects on cellular hydration and membrane integrity, consistent with prior reports showing that drought stress causes oxidative damage and compromises photosynthetic activity (Farooq et al. 2018; Tejaswini et al. 2025). The duration and extent of water stress determine the chlorophyll level in plants (Kpyoarissis et al. 1995). Conversely, the marked enhancement in proline and total soluble sugars (TSS) content observed in this study highlights their crucial roles in osmotic adjustment and stress-induced metabolic reprogramming (Bates et al. 1973; Kumar et al. 2011). These metabolites contribute to cell turgor maintenance, enzyme stabilization and reactive oxygen species (ROS) detoxification under dehydration stress.

The parallel reductions in NDVI and CTD provide physiological evidence of impaired photosynthetic capacity and diminished transpiration cooling. Such shifts have also been recorded in drought stressed legumes, where lower NDVI and CTD values signify reduced canopy vigor and stomatal conductance (Karimizadeh et al. 2021). Among the tested genotypes, IPU9612, IPU243 and IC546466 maintained comparatively higher yield and physiological resilience across environments, reflecting their inherent drought tolerance (Hossain et al. 2010). Genotypes IPU9612 and IPU941, characterized by higher SPAD and CTD values, likely possess more efficient photosynthetic maintenance mechanisms and better heat dissipation, attributes that favour survival under drought (Hayatu et al. 2011; Mafakheri et al. 2010). In contrast, the poor performance of IC436635 and IC436621 identifies them as drought-sensitive entries with limited adaptive capability.

Molecular profiling using SSR markers revealed substantial genetic diversity among 60 black gram genotypes. The 389 alleles detected across 130 loci, with an average of approximately 3 alleles per locus and a high mean PIC value (0.57), reveal comprehensive allelic richness. Similar allelic variation was reported in earlier black gram and mung bean studies, supporting SSRs as robust genetic markers for diversity estimation (Souframanien and Gopalakrishna 2004; Somta et al. 2009). The model-based STRUCTURE analysis categorized the black gram genotypes into two distinct populations, corroborating the UPGMA clustering results that revealed clear genetic differentiation among the genotypes. This

concordance suggests the presence of well-defined genetic pools within the germplasm, which can be strategically exploited to facilitate hybridization between divergent groups, thereby maximizing heterosis and recombination potential in breeding programs. In a related study, Singh et al., 2022 also identified four subpopulations among black gram genotypes using SNP markers, further supporting the genetic stratification observed in this species.

The marker trait association analysis using GLM method identified multiple significant SSR trait linkages under both control and stress regimes, reinforcing the polygenic nature of drought tolerance. Multiple markers influencing a single trait per chromosome highlight the cumulative contributions of major and minor alleles, while markers linked to multiple traits demonstrate pleiotropic effects. Such pleiotropic loci hold substantial promise for marker-assisted selection (MAS) to enhance production and productivity (Gupta et al. 2020).

Under control conditions, markers such as VmgEST72, VmGEN4 and VmgEST77 were found to be associated with traits like CTD, MSI and SPAD, indicating the involvement of genomic regions regulating canopy temperature maintenance, membrane stability and chlorophyll retention. Notably, VmgEST72 encodes a TBCC domain-containing protein 1-like, while VmgEST77 corresponds to a late embryogenesis abundant (LEA) protein D-34. LEA proteins are well known for their protective roles during dehydration by stabilizing proteins and membranes (Hong-Bo et al. 2005), and their association with these traits supports their contribution to cellular resilience under water deficit.

Under stress, associations involving VmGEN58, VmgEST2 and VmgEST77 linked to key physiological parameters (RWC, MSI and SPAD). These consistent associations suggest the presence of genomic regions governing drought tolerance and water balance regulation (Singh et al. 2021). VmgEST2 encodes a plant defensin (D1) protein, which functions in pathogen defense and stress response, suggesting a role in enhancing resilience under drought stress (Domingo et al. 2024). The recurrent association of VmgEST77 under both control and stress conditions highlights the importance of LEA-mediated protection in sustaining physiological stability across environments (Ban QiaoYing et al 2008). These findings indicate that drought tolerance is not controlled by a single pathway but rather by coordinated regulation of stress protection, ROS scavenging and cellular integrity.

Yield-related traits showing consistent associations with VmGEN19, VmEST5 and VmEST36 further indicate that drought tolerance and productivity share interconnected genetic regulation driven by overlapping pathways influencing resource use efficiency (Varshney et al. 2014). VmEST5 encodes an SCY1-like protein 2" HEAT repeat", which may be involved in protein-protein interactions and intracellular transport (Yao et al. 2022), while VmEST36 encodes a probable glycosyltransferase, suggesting a role in carbohydrate metabolism and biomass allocation (Tang et al. 2022). VmGEN19, VmEST5 and VmEST36 may therefore contribute indirectly to yield maintenance under stress by influencing assimilate partitioning, metabolic adjustment and structural stability.

While markers VmgEST10, VmEST5, VmgEST2, VmgEST75 and VmgEST77 detected under both control and stress, could represent constitutive loci functioning across environments, a valuable attribute for

marker-assisted breeding (Xu and Crouch 2008). Among markers used, VmgEST10 encodes a bHLH68-like transcription factor, which may regulate stress- responsive gene expression, while VmEST75 is annotated as beta-conglycinin alpha subunit 2, a seed storage protein associated with carbon and nitrogen reserve accumulation (Hu et al. 2025; Sicilia et al. 2024). The stability of these markers suggests that they may contribute to both physiological regulation and adaptive responses under drought, making them promising candidates for molecular breeding pipelines aimed at improving resilience in black gram.

Overall, the combined morpho-physiological, yield and marker-association data underscore that drought tolerance in these genotypes involves multi-locus and multi-trait coordination. These functions of the associated EST markers further suggest that the trait response mediated by many genes and single gene with pleotropic effects are involved in imparting tolerance for complex trait like drought. These markers are responsible for activating different metabolic pathways such as dehydration protection, redox homeostasis, membrane stabilization, transcriptional regulation and biomass partitioning. Together, these loci provide a strong foundation for marker-assisted selection aimed at improving drought resilience and productivity in black gram.

Conclusion

Collectively, the results provide an integrated picture of how black gram genotypes respond to contrasting moisture regimes at morphological, physiological and molecular levels. Genotypes such as IC546466, IPU9612, IPU243, IC426766 and IC331232 emerged as promising sources that combine high yield stability with favourable physiological responses and desirable molecular profiles, making them strong candidates as parental lines for drought-prone environments. Markers linked with different pathways such as VmgEST10, VmEST5, VmgEST2, VmgEST75 and VmgEST77 is found to be closely associated with drought tolerance related traits. The SSR markers identified in association with key drought-responsive traits and yield components can serve as genomic anchors for further validation, fine mapping and QTL dissection. Together, these findings establish a strong foundation for implementing marker-assisted selection and ultimately genomic-assisted breeding strategies aimed at enhancing drought tolerance and yield stability in black gram.

Declarations

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Author contributions

All authors contributed to the conception and design of the study. Conceptualization, planning experiment, selection of material preparation, and execution were carried out by B. Sarkar. Experimentation, data recording, tabulation, original manuscript writing and editing were performed by B. Sarkar, R. Mythily. Statistical analysis was conducted by B. Sarkar and A. Bharath Kumar. Reviewing and editing of the manuscript were carried out by B. Sarkar, N. Jyothi Lakshmi, K. Salini, H.B. Santhosh and V.K. Singh. Overall coordination and fund management for the experiment were handled by B. Sarkar and V.K. Singh. All authors have read and approved the submitted version of the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal interests that could have appeared to influence the reported work.

Data availability

The datasets generated during this study are available from the corresponding author upon reasonable request.

Ethical compliance

All plant experiments were conducted in accordance with relevant institutional, national and international guidelines and regulations. All authors have approved the manuscript for publication.

Supplementary material

The supplementary materials associated with this article are provided as separate online links.

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Figures

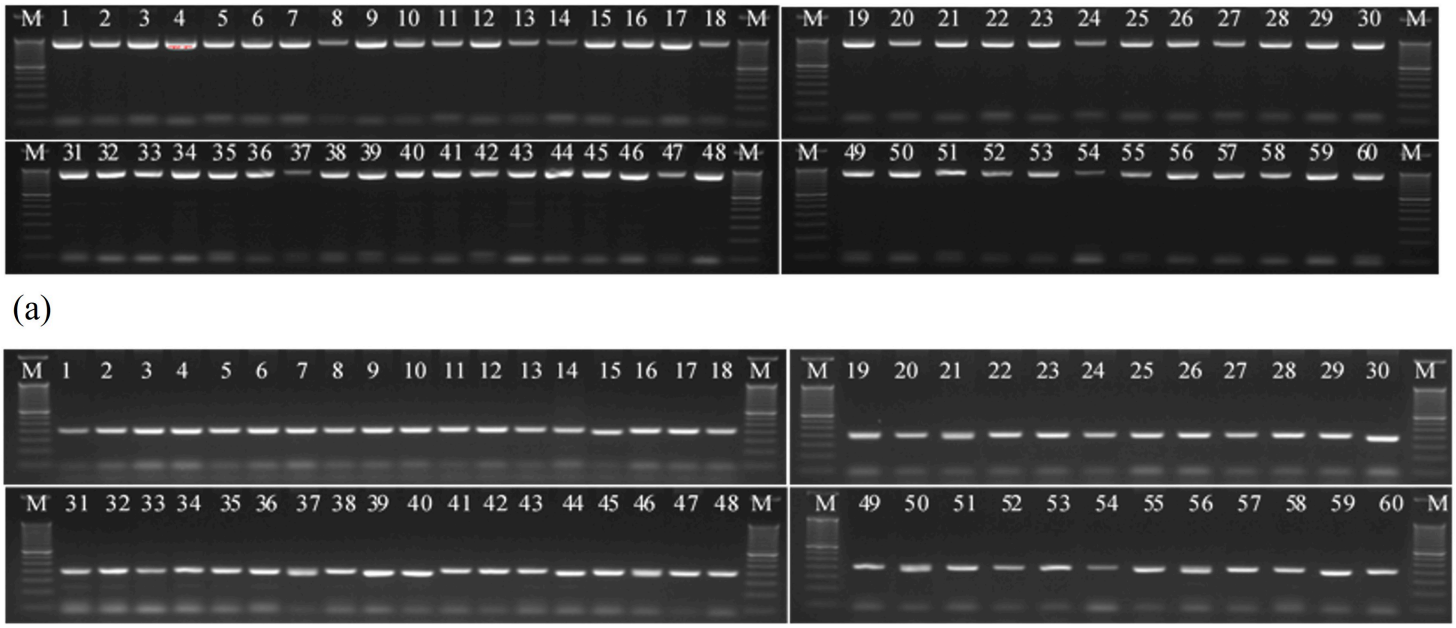

Fig. 1 SSR profiles generated from 60 black gram genotypes using primers VmgEST79 (a) and VmgEST61 (b). lane M: 50bp DNA ladder

Figure 1

See image above for figure legend.

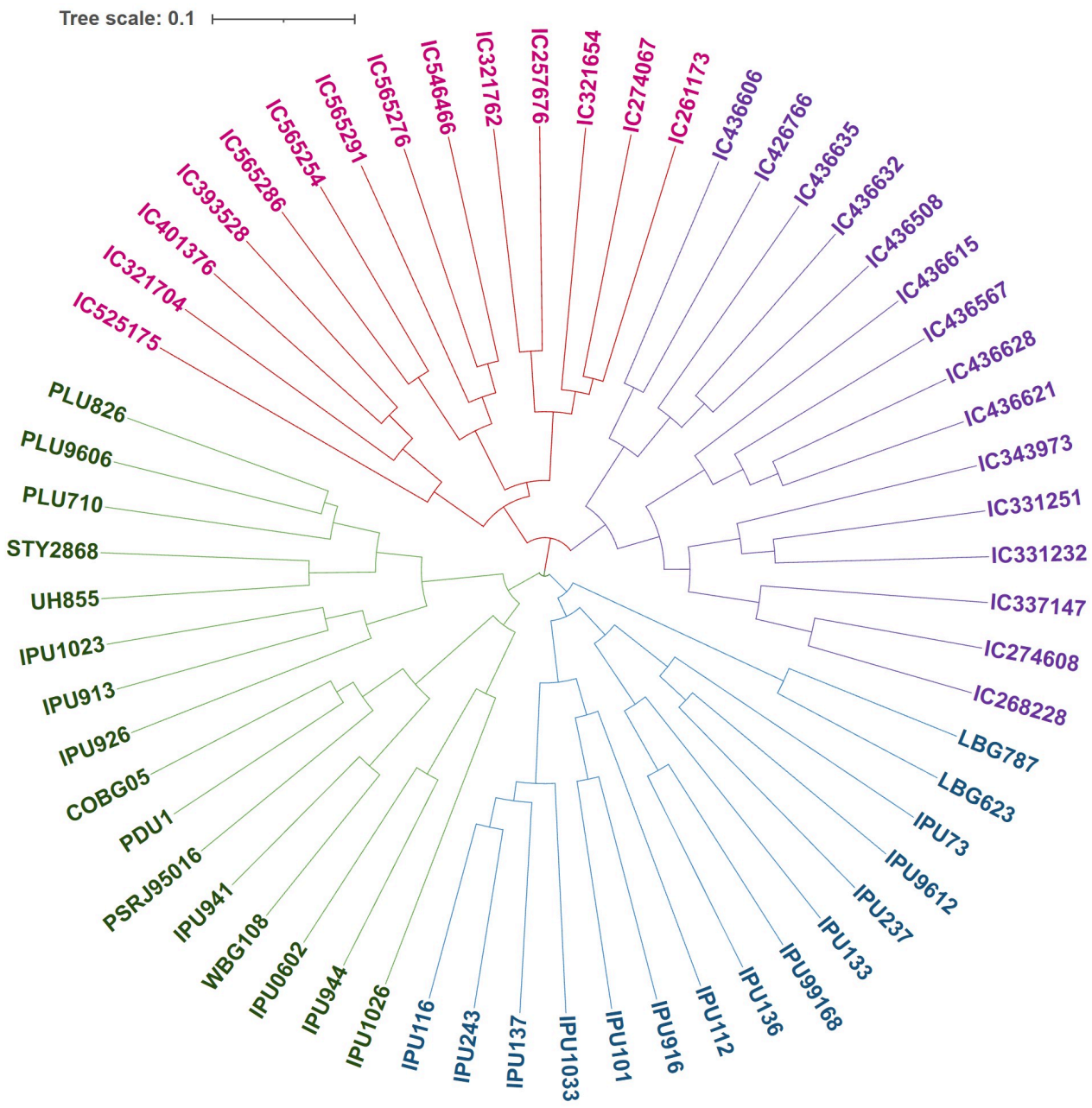

Fig. 2 Phylogenetic tree of 60 black gram genotypes using 130 SSR data constructed by UPGMA algorithm (Nei, 1983)

Figure 2

See image above for figure legend.

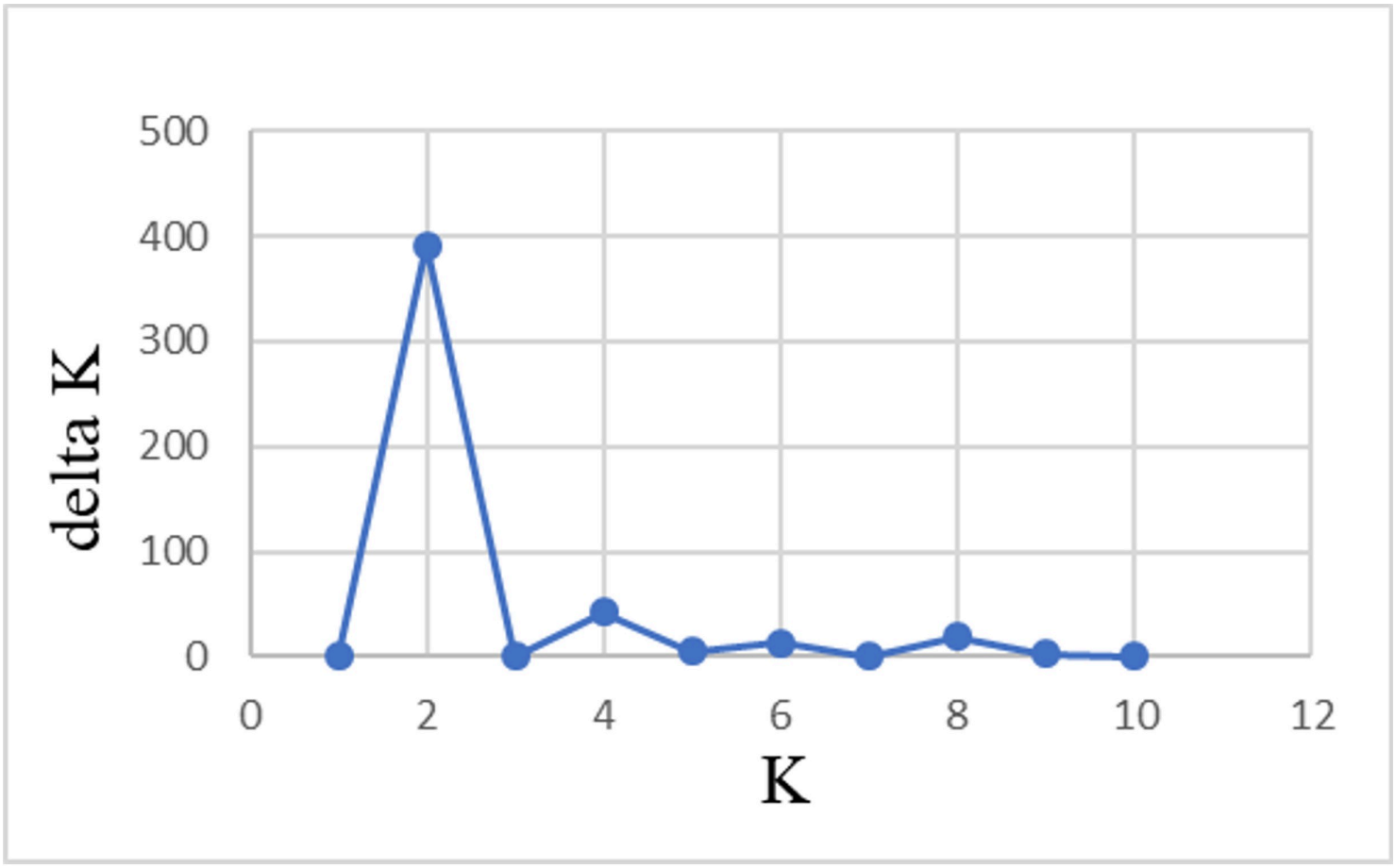

Fig. 3 Estimated ΔK values of black gram genotypes (Evanno et al., 2005)

Figure 3

See image above for figure legend.

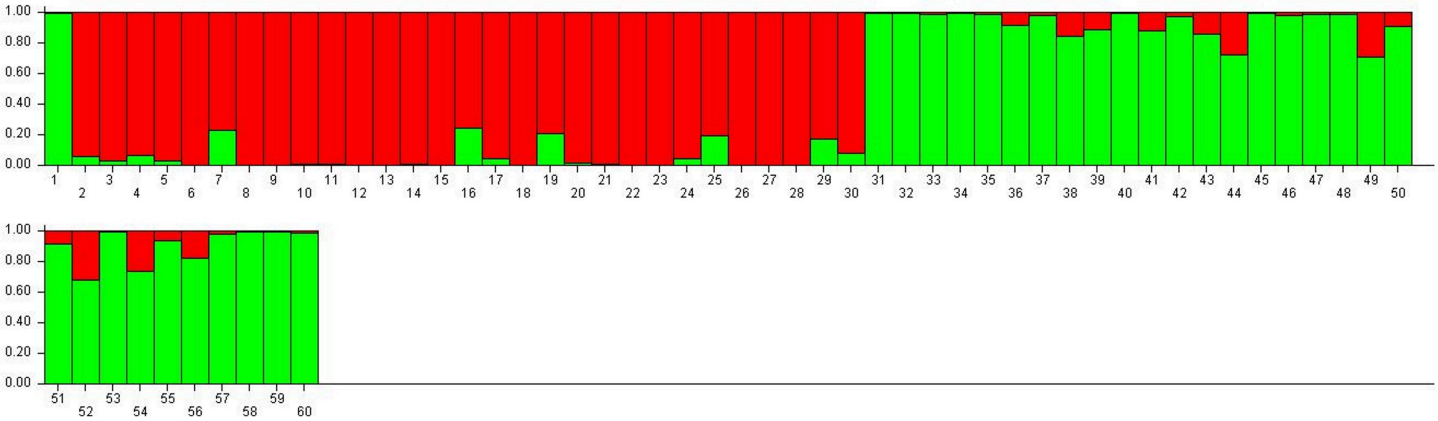

Fig. 4 Estimated population structure of 60 black gram genotypes using structure software

Figure 4

See image above for figure legend.

Supplementary Files

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