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Identification of new alleles resistant to moisture stress in mutant lines of wheat genotypes developed by chemical mutagenesis through drought-specific ISSR markers

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Water stress is a significant global issue that severely limits crop production worldwide, and recent changes in the global climate have exacerbated the problem. Wheat crops are especially vulnerable to water shortages, which result in decreased yield and production. Mutation breeding offers a potential alternative approach for developing crops that are important for agriculture. It can enhance specific traits without altering entire genotype. In this study, ISSR markers were employed to evaluate genetic variation and diversity at the molecular level among selected M_3 mutant lines and wild-type wheat genotypes after chemical mutagenesis in response to moisture stress during 2022–2023 *rabi* season at SVPUA&T, Meerut, India. Out of 15 ISSR primers, 12 showed successful amplification across both genotypes. In HD-3226, 50 alleles and 585 amplified bands were recorded across 13 ISSR loci. PIC values ranged from 0.21 to 0.81, with an average of 0.47 for EMS and 0.39 for SA. The resolving power ranged between 2.33 and 9.56, while the marker index ranged from 0.59 to 3.95. Nine primers exhibited 100% polymorphism, and genetic similarity ranged from 0.40 to 0.88. UPGMA clustering grouped mutant lines into five clusters with genetic distances ranges from 0.54 to 1.00. The greatest dissimilarity (0.404) was observed between mutant 0.5% EMS (15% PEG, P-41) and 0.75% EMS (15% PEG, P-63) whereas the highest similarity (0.880) was between 0.04% SA (15% PEG, P-36) and 0.25% EMS (15% PEG, P-19). In HI-1620, 41 alleles and 273 amplified bands were produced. PIC values ranged from 0.00 to 0.89, with averages of 0.35 (EMS) and 0.42 (SA). Resolving power varied from 1.40 to 7.00, and marker index from 0.30 to 2.75. Eight primers showed 100% polymorphism. Genetic similarity ranged from 0.411 to 0.823. UPGMA dendrogram formed four clusters with genetic distances between 0.50 and 0.97. M_3 mutant lines 0.02% SA (15% PEG, P-28) and 0.25% EMS (15% PEG, P-4) were found to closely dissimilar (0.411) to each other while highest genetic similarity (0.823) was detected between mutant lines 0.02% SA (15% PEG, P-4) and 0.25% EMS (15% PEG, P-4). Our study found that ISSR markers, with their high PIC and resolving power, are highly effective at distinguishing closely related wheat mutant lines. Furthermore, identified M_3 mutants represent promising resources for reverse genetics and functional genomics approaches to improve drought tolerance in wheat.

Keyword: Genetic diversity, Induced mutation, Marker Index (MI) and polymorphism percentage, PIC, Resolving power, Water stress, Wheat

Wheat (*Triticum aestivum* L.*)* is member of the family Poaceae. It is most diverse and agronomically important family of the plant kingdom[1]. As a *Rabi* season crop, wheat has played an essential role in maintaining food grain production in India. Wheat provides over 20% of the total calories and protein in the human diet[2]. It is rich in protein and dietary fiber, with grain containing 8-15% protein and flour containing 8-13%, along with 60-80% starch[3]. In India, bread wheat occupies 86% of the total wheat cultivation area, while durum wheat accounts for only 12%[4]. Global climate change significantly threatens the sustainability of agricultural production[5]. Among the various environmental stresses, drought stands out as the most critical factor limiting plant growth and crop yields due to water shortages in both managed

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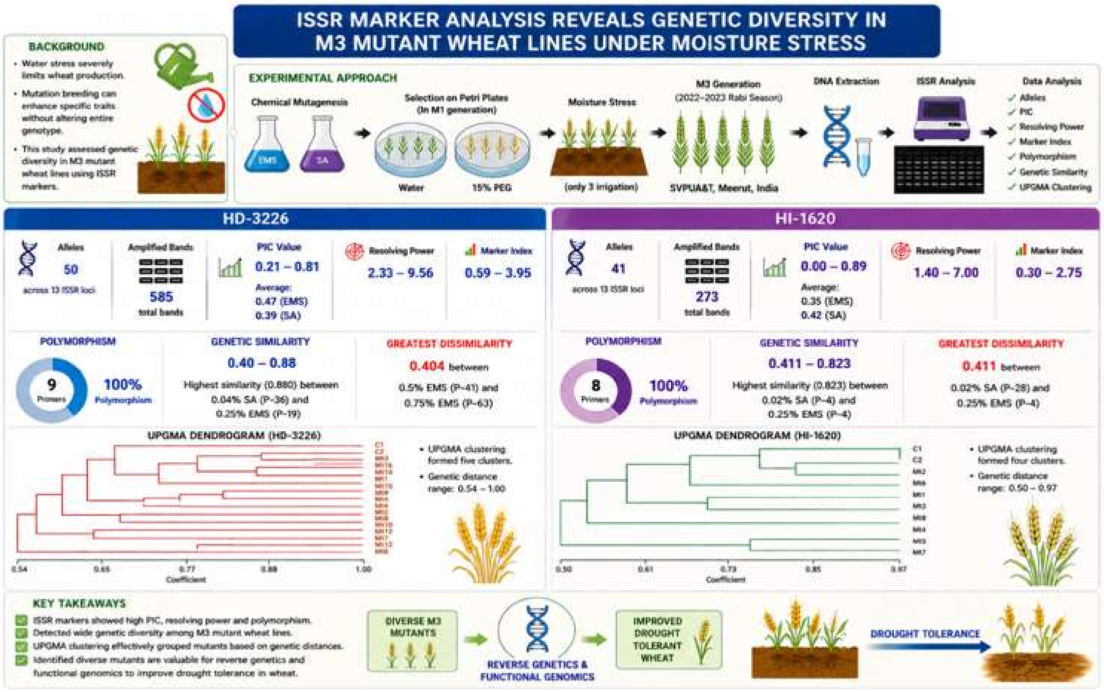
Abbreviations: EMS, Ethyl Methane Sulphonate; ISSR, Inter-Simple Sequence Repeats; Mt, Mutant; PCR, Polymerase Chain Reaction; PIC, Polymorphic Information Content; Rp, Resolving Power; SA, Sodium Azide; WT, Wild Type

Suppl. data available on respective page of NOPR



Graphical abstract

cultivated lands and natural ecosystems. Wheat production, in particular, suffers from multiple abiotic stresses, leading to substantial yield reductions[6]. Major effects of drought stress include reduced water uptake, stomatal closure, increased transpiration rates, and excessive production of reactive oxygen species[7]. This stress can affect any stage of wheat growth and varies based on the local environment. Water stress tolerance is the ability of a plant to withstand and survive in conditions where water is scarce. Although this trait is found in almost all plants, the level of tolerance can differ greatly between species and even among different plants of same species[8].

In recent years, initiatives to improve wheat have focused on utilizing induced mutation as a strategy for cereal breeding[9]. Mutagenesis can occur naturally or be induced by exposure to mutagens, such as chemicals like sodium azide and ethyl methane sulphonate (EMS), or through radiation like X-rays and gamma rays[10,11]. EMS mutagenesis induces a high frequency of point mutations[12,13]. It has been successfully applied to a variety of crops, including oat, barley, maize, rice and wheat[14]. On the other hand, sodium azide (NaN_3) is recognized as one of the most effective chemical mutagens for plants[15]. Sodium azide has been shown to be a mutagen in various plants, including wheat, maize, rice, chickpea, fenugreek, sunflower, rapeseed, and tomato[16]. By integrating the extensive variation from mutagenized populations with modern screening methods, traits that are challenging to detect through traditional breeding are now being developed and examined at the molecular level[10]. Genetic diversity is vital for advancing crop species like wheat. Crop improvement initiatives largely rely on the genetic variation available in existing germplasm[17]. Today, a number of DNA-based markers are utilized to precisely evaluate genetic diversity in plant species. ISSR (inter-simple sequence repeats) are non-coding DNA regions that do not encode proteins but are useful for DNA fingerprinting due to their distinctive placement among coding genes[18]. Polymorphic information content measures the effectiveness of a marker in detecting mutations, which depends on the number of detectable alleles and their frequency distribution within a selected population. A high PIC value indicates a high mutation frequency; while a low PIC value reflects a low mutation frequency at a given marker site and resolving power (Rp) refers to the capacity of a molecular marker to distinguish among different genotypes and its effectiveness in revealing genetic variation within a population. In plant breeding programs, genetic similarity data from ISSRs help researchers select superior mutant plantlets for improvement or develop new cultivars through mutagenesis techniques[19]. The aim of present investigation was identification of variation at molecular level in selected M_3 mutant lines of wheat genotypes after chemical mutagenesis towards moisture stress. These mutagenic lines of wheat were developed with improve traits like plant height, yield, growth period, and water stress tolerance.

Materials and Methods

Total fifteen ISSR markers were utilized for screening of twenty-four mutant plants generated through chemical treatment of EMS and SA along with control-wild type plants of two HD-3226 and HI-1620 wheat genotypes. All process and methodology to develop mutant population was described in detail in Rani *et al.,*[20]. The selection of mutants plants were based on morphologically parameters i.e. plant height, leaf length and width, spike length, maturity and test weight etc. of both wheat genotypes in M_2 generation (2021-2022) and then selected mutants screened to identify mutation along with their controls in M_3 generation (2022-2023) using fifteen drought specific ISSR markers. Two wheat genotypes consisted one positive (HI-1620) and one negative (HD-3226) check along with mutant plants were grown at technology research field while molecular work was performed in the genomics laboratory of the agricultural biotechnology department, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (U.P.), India. All ISSR markers were chosen for their reproducibility, amplification and scorable patterns of bands. ISSR markers for genetic diversity analysis involves following steps: 1. Isolation of genomic DNA and quantification, 2. DNA amplification using PCR with ISSR primers and 3. Determining the PCR amplified products

Genomic DNA isolation and Purification

CTAB method (Doyle and Doyle) was used to extract genomic DNA from fresh leaves of regenerated normal and M_3 mutant wheat plantlets, 2 g of fresh leaf tissue was ground into a fine powder (with liquid nitrogen) in order to isolate DNA[21]. After that, mixture was put into a centrifuge tube and 2 mL of CTAB extraction buffer was added. The material was periodically shaken and incubated for 1 h at 65°C. After that, an equal volume of chloroform: isoamyl alcohol (24:1) was added, and the mixture was gently stirred for a duration of 15 to 20 min. The tubes were centrifuged at 11,000 rpm for 10 min, after which aqueous phase was transferred to a fresh tube and re-extracted with chloroform: isoamyl alcohol. Chilled isopropanol (0.6 volume) was added, and then mixture was kept at -20°C for 30 min. The tubes were centrifuged again at 11,000 rpm for 10 min. After centrifugation, supernatant was discarded, and pellet was washed with 70% ethanol, centrifuged at 10,000 rpm for 5 min at 4°C. The pellet was air-dried and dissolved in 40 µL of molecular-grade water. Finally, 2.0 µL of RNase A (10 mg/ml) was added to the DNA solution to degrade RNA. The quality and quantity of the isolated DNA from each sample were analyzed using a Nanodrop device.

DNA amplification using PCR with ISSR primers

DNA amplification was performed using fifteen ISSR primers (Table 1) synthesized by Bangalore Genei Pvt. Ltd. The reactions were carried out in a total volume of 15 µL containing optimized concentrations of specific components. Each reaction mixture consisted of 1.6 µL of 10X Assay Buffer with 15.0 mM $MgCl_2$, 1.0 µL of 1.0 mM dNTPs Mix, 0.5 µL of Taq DNA Polymerase (1U/µL), 1.0 µL of 5.0 mM primer, 1.2 µL of template DNA (25 ng/µL), and 9.7 µL of Milli-Q water. To maintain consistency and reduce pipetting errors, a master mix was prepared containing all components except template DNA for the required number of reactions. The master mix was briefly centrifuged and distributed into 0.2 mL thin-walled PCR tubes, followed by individual addition of template DNA to each tube. Annealing temperatures for each primer were calculated using the formula $Tm = (A+T) \times 2 + (G+C) \times 4$, and the actual annealing temperature was set 3-5°C below the calculated melting temperature.

PCR amplification was carried out in a HIMEDIA Prima-Duo Thermal Cycler using a standardized thermal cycling program. The protocol initiated with an initial denaturation at 95°C for 5 min, followed by 35 cycles comprising denaturation at 94°C for 1 minute, primer-specific annealing for 30 seconds, and extension at 72°C for 1 minute. Following the cycling steps, a final extension was performed at 72°C for 7 min to ensure complete synthesis of all amplification products. The amplified products were subsequently maintained at 4°C for 1 h until further analysis.

Determining the PCR amplified products

2.0% (*w/v*) agarose gel with 1x TAE buffer was used for separation of amplified product. Amplified DNA samples were mixed with a loading dye in a 5:1 ratio and at 94 volts for 30 min. Gel was viewed

Table 1 — List of ISSR primers and their sequences used for the analysis of wheat germplasm

S. No.	Primer Code Name	Primer Sequence 5′-3′	Position in the genome	References
1.	UBC-810	GAGAGAGAGAGAGAGAT	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Hajiyev *et al*., 2021[36]
2.	UBC-812	GAGAGAGAGAGAGAGAA	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Hajiyev *et al*., 2021[36]
3.	UBC-814	CTCTCTCTCTCTCTCTA	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Son *et al*., 2013[40]
4.	UBC-815	CTCTCTCTCTCTCTCTG	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Khaled *et al*.,2015[39]
5.	UBC-823	TCTCTCTCTCTCTCTCC	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Sen *et al*., 2017[5]
6.	UBC-826	ACACACACACACACACC	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Khaled *et al*.,2015[39]
7.	UBC-834	AGAGAGAGAGAGAGAGCT	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Hajiyev *et al*., 2021[36]
8.	UBC-840	GAGAGAGAGAGAGAGACT	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Son *et al*., 2013[40]
9.	UBC-845	CTCTCTCTCTCTCTCTAG	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Sen *et al*., 2017[5]
10.	UBC-848	CACACACACACACACAAG	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Son *et al*., 2013[40]
11.	UBC-852	TCTCTCTCTCTCTCTCGA	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Sen *et al*., 2017[5]
12.	UBC-853	TCTCTCTCTCTCTCTCAT	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Sen *et al*., 2017[5]
13.	UBC-855	ACACACACACACACACCT	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Khaled *et al*.,2015[39]
14.	UBC-876	GATAGATAGAGAGACA	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Khaled *et al*.,2015[39]
15.	UBC-881	GGGTGGGGTGGGGTG	1A,1B,1D,2A,2B,2D,3A.3B,3D,4A,4B,4D,5A,5B,5D,6A,6B,6D,7A,7B,7D	Khaled *et al*.,2015[39]

Note: Selected ISSR markers were screened to determine their chromosomal locations using BLASTn on NCBI. The results revealed that all ISSR primers were successfully mapped to chromosomes, as described in the table. BLAST analysis showed 100% similarity, confirming that all primers are located on the chromosomes of wheat cultivars. These selected primers were then used for genetic diversity analysis and further molecular analysis in mutants.

to identify variations by using a UVITECH (Cambridge) Gel Documentation System.

Statistical Molecular analysis

Polymorphic band of ISSR markers where score as 0 for their absence and 1 for presence absence at a specific locus. Various indicators such polymorphic information content (PIC), resolving power (Rp), marker index, polymorphism percentage (%), number of amplified bands, total number of alleles, monomorphic and polymorphic alleles were used to check and compare the efficiency of molecular markers. Variation was depicted with phylogenetic analysis with available software NTSYS version 2.1[22]. Pairwise genetic similarities among mutant lines were calculated using Jaccard's coefficient method[23]. Genetic similarity matrix was analysed using unweighted pair group method with arithmetic mean (UPGMA)[24]. Milbourne *et al*.[25] was proposed a formula for calculation PIC value and it was calculated by following formula:

$$PIC= 1-\sum Xi^2$$

Where, Xi = relative frequency of the 'i' allele of ISSR loci. The resolving power was calculated by Provost and Wilkinson's formula[26]:
Resolving power (Rp): $Rp=\Sigma I b$

Where, I b represents band informativeness, and
$I b=1-[2\times(0.5-p)]$

Where, p represents proportion of genotypes including band. Marker Index (MI) was introduced by Powell *et al.*[27] to measure the efficiency of a molecular marker system in detecting polymorphism. The formula is:

MI= PIC value × Number of Polymorphic Alleles

Where, PIC indicates the informativeness of a marker and number of polymorphic alleles represents how many alleles are polymorphic for each marker.

Results

Characterization of selected M_3 mutants lines of both wheat genotypes using ISSR markers

Total fifteen ISSR primers were utilized for molecular characterization of twenty four mutant plants in two wheat genotypes. Twelve primers were showed polymorphic bands (UBC-810, UBC-814, UBC-815, UBC-823, UBC-834, UBC-840, UBC-845, UBC-848, UBC-852, UBC-853, UBC-855 and UBC-881), one primer showed monomorphic band (UBC-826) but two primers was not amplified in all plants of both genotypes. In HD-3226 genotype, total of 50 alleles and 585 total numbers of amplified bands were produced in thirteen ISSR loci. Maximum allele was observed with primer UBC-840 and UBC 845 while wild type of both wheat genotypes showed lesser genetic diversity in compare to twenty four M_3 mutant lines which produced wide range of diversity based on frequency of alleles (Fig. 1). Polymorphic information content values range from 0.21 to 0.81 and UBC 853 showed lowest PIC value (0.21) while UBC 881 showed maximum PIC (0.81) and average PIC value across all loci analysed were 0.47 (EMS) and 0.39 (SA) (Table 2). Resolving power ranges from 2.33 to 9.56 for the primer UBC-834 and UBC-840, respectively. Marker index values ranges from 0.59 (UBC 823) to 3.95 (UBC 845) and nine primers (UBC 814, 815, 834, 845, 848, 852, 853, 855 and 881) were showed highest (100%) polymorphic rate while UBC 823 showed lowest polymorphic rate (50%) (Fig. 2). In HI-1620 genotype, total of 41 alleles and 273 total numbers of amplified bands were produced in thirteen ISSR loci. Maximum alleles were observed with primer UBC-810 and UBC-845 while wild type of both wheat genotypes showed lesser genetic diversity in compare to twenty four M_3 mutant lines which produced wide range of diversity based on frequency of alleles. PIC ranges from 0.00 to 0.89 and

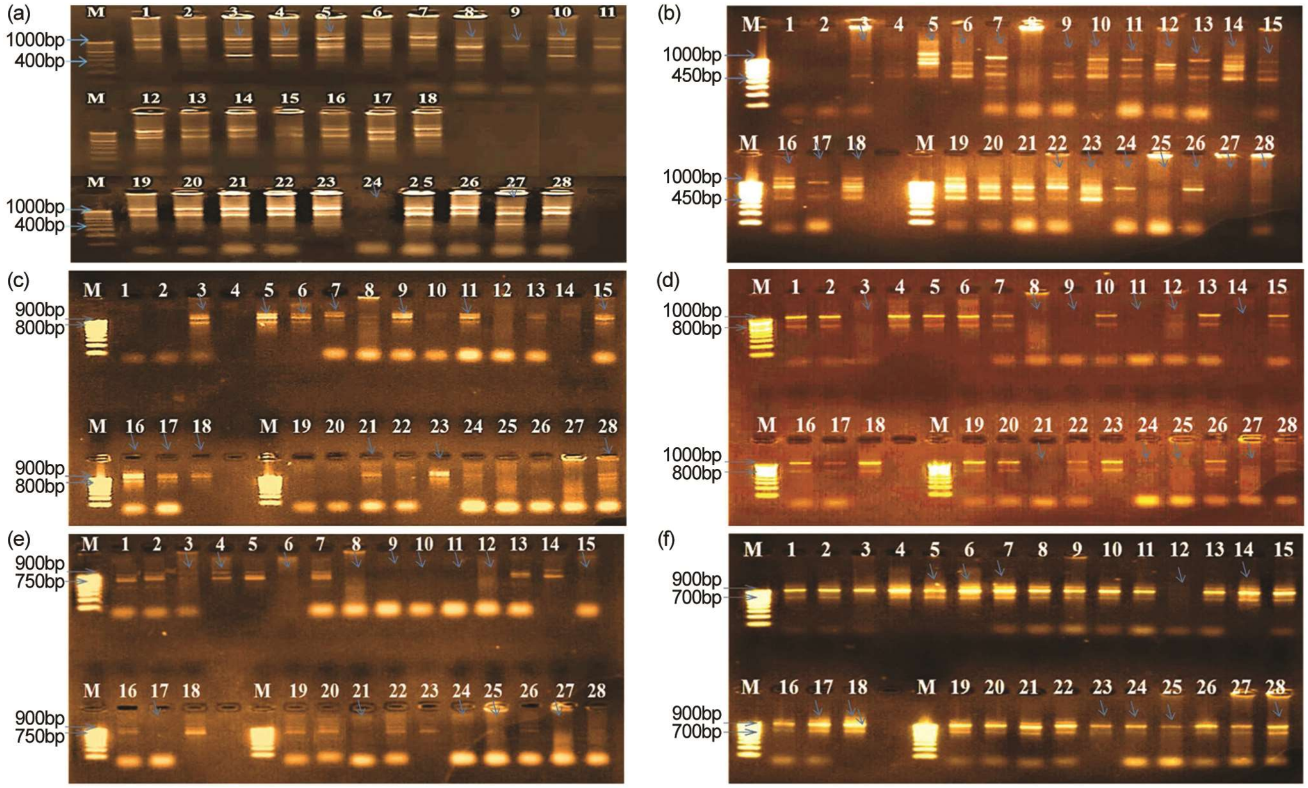


Fig. 1 — ISSR profiles of 28 mutant lines of wheat genotypes (1-28). PCR amplification products were represented on 2% agarose gel by sizes ranging from 400 to 1,000 bp. The blue Arrows indicate the the variations in mutant lines. (a) UBC840; (b) UBC845; (c) UBC848; (d) UBC852; (e) 881; and (f) UBC855

Table 2 — Primer code, number of amplified bands, mono and polymorphic alleles, PIC value and resolving power of 18 wheat normal and mutant plant of HD 3226 variety

Primers	Total Number of alleles	Number of amplified bands	Polymo - rphic alleles	Monom - orphic alleles	Polymorphism %	PIC value		Marker index	Resolving power
						EMS	SA		
UBC 810	5	74	3	2	60.00	0.22	0.27	0.73	8.22
UBC 814	3	25	3	0	100.00	0.72	0.34	1.59	2.78
UBC 815	4	54	4	0	100.00	0.38	0.36	1.48	6.00
UBC 823	4	57	2	2	50.00	0.25	0.34	0.59	6.33
UBC 826	2	36	0	2	0.00	0.00	0.00	0.00	4.00
UBC 834	3	21	3	0	100.00	0.73	0.39	1.68	2.33
UBC 840	6	86	4	2	66.67	0.31	0.31	1.24	9.56
UBC 845	6	51	6	0	100.00	0.80	0.52	3.96	5.67
UBC 848	3	31	3	0	100.00	0.66	0.79	2.17	3.44
UBC 852	2	23	2	0	100.00	0.59	0.47	1.06	2.55
UBC 853	4	56	4	0	100.00	0.46	0.21	1.34	6.22
UBC 855	3	45	3	0	100.00	0.26	0.37	0.95	5.00
UBC 881	5	26	5	0	100.00	0.69	0.81	3.75	2.89
Total	50	585	42	8	Average	0.47	0.39	1.58	4.99

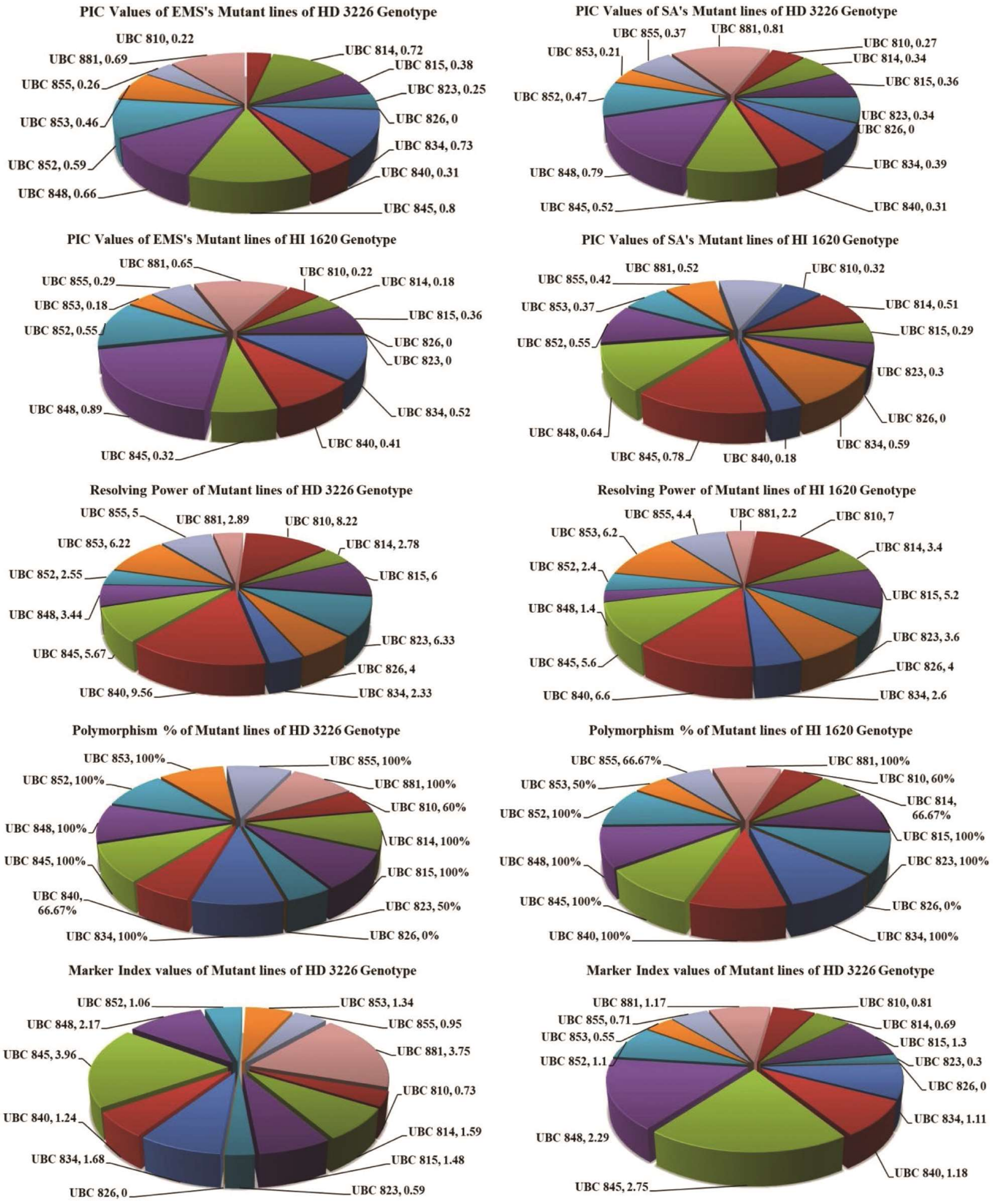
UBC-823 showed lowest PIC value (0.00) while UBC 848 showed maximum PIC (0.89) with average PIC value across all loci analysed were 0.35 (EMS) and 0.42 (SA). Resolving power ranges from 1.40 to 7.00 for primer UBC-848 and UBC-810, respectively. Marker index values ranges from 0.30 (UBC 823) to 2.75 (UBC 845) (Table 3). Eight primers (UBC 815, 823, 834, 840, 845, 848, 852 and 881) were showed highest (100%) polymorphic rate while UBC 853 showed lowest polymorphic rate (50%). The significant PIC value indicated that primers were capable of effectively differentiating between various closely related wheat genotypes.

M- Marker (100 bp) and P- Serial numbers of mutant plant

Genotype HD- 3226 (Lane 1 to 18):- 1. Control with water, 2. Control with 15% PEG, 3. 0.25% EMS water P-21, 4. 0.25% EMS water P-10, 5. 0.25% EMS 15% PEG P-19, 6. 0.5% EMS water P-49, 7. 0.5% EMS 15% PEG P-41, 8. 0.75% EMS 15% PEG P-63, 9. 0.75% EMS 15% PEG P-67, 10. 1% EMS 15% PEG P-19, 11. 1% EMS 15% PEG P-25, 12. 0.02% SA water P-4, 13. 0.02% SA 15% PEG P-28, 14. 0.02% SA 15% PEG P-55, 15. 0.04 SA water P-7, 16. 0.04% SA 15% PEG P-2, 17. 0.04% SA 15% PEG P-43, 18. 0.04% SA 15% PEG P-36, Genotype HI-1620 (Lane 19 to 28):- 19. Control with water, 20. Control with 15% PEG, 21. 0.25% EMS water P-11, 22. 0.25% EMS 15% PEG P-4, 23. 0.25% EMS 15% PEG P-45, 24. 1% EMS 15% PEG P-21, 25. 0.02% SA 15% PEG P-28, 26. 0.02% SA 15% PEG P-4, 27. 0.08% SA 15% PEG P-1, 28. 0.08% SA 15% PEG P-2.

Genetic similarity among M_3 mutant lines of two wheat genotypes and Cluster analysis

Allele frequency data were used to estimate genetic similarities among M_3 mutant lines of two wheat genotypes. Jaccard's coefficient method was used for estimation of pairwise genetic similarity. The distribution pattern of the similarity coefficient values are shown in (Figs. 3 & 4). Mutant lines of genotype HD-3226 Mt5-0.5% EMS (15% PEG, P-41) and Mt6-0.75% EMS (15% PEG, P-63) was found to closely dissimilar (0.40) to each other. Highest genetic similarity (0.88) was detected between mutant lines Mt16-0.04% SA (15% PEG, P-36) and Mt3-0.25% EMS (15% PEG, P-19) (Table 4). In HI-1620 genotype, mutant lines Mt5-0.02% SA (15% PEG, P-28) and Mt2-0.25% EMS (15% PEG, P-4) were found to closely dissimilar (0.411) to each other. Highest genetic similarity (0.823) was detected between mutant lines Mt6-0.02% SA (15% PEG, P-4) and Mt2-0.25% EMS (15% PEG, P-4 (Table 5). A dendrogram was constructed using the UPGMA method based on the presence (1) and absence (0) of different alleles in mutant lines generated by 13 ISSR markers, effectively grouping the mutant lines of both wheat genotypes into distinct clusters. In HD-3226 genotype, dendrogram derived from this analysis was depicted as it contain five clusters at 0.65 coefficient, cluster I, II, III, IV and V. Cluster I contains one



PIC Values of EMS's Mutant lines of HD 3226 Genotype

PIC Values of SA's Mutant lines of HD 3226 Genotype

PIC Values of EMS's Mutant lines of HI 1620 Genotype

PIC Values of SA's Mutant lines of HI 1620 Genotype

Resolving Power of Mutant lines of HD 3226 Genotype

Resolving Power of Mutant lines of HI 1620 Genotype

Polymorphism % of Mutant lines of HD 3226 Genotype

Polymorphism % of Mutant lines of HI 1620 Genotype

Marker Index values of Mutant lines of HD 3226 Genotype

Marker Index values of Mutant lines of HD 3226 Genotype

Fig. 2 — PIC values, Polymorphism %, Resolving power and Marker index values of mutant lines of both wheat genotypes

Table 3 — Primer code, number of amplified bands, mono and polymorphic alleles, PIC value and resolving power of 10 wheat normal and mutant plant of HI-1620 variety

Primers	Total Number of alleles	Number of amplified bands	Polymo - rphic alleles	Monom - orphic alleles	Polymorphism %	PIC value		Marker index	Resolving power
						EMS	SA		
UBC 810	5	35	3	2	60.00	0.22	0.32	0.81	7.00
UBC 814	3	17	2	1	66.67	0.18	0.51	0.69	3.40
UBC 815	4	26	4	0	100.00	0.36	0.29	1.30	5.20
UBC 823	2	18	2	0	100.00	0.00	0.30	0.30	3.60
UBC 826	2	20	0	2	0.00	0.00	0.00	0.00	4.00
UBC 834	2	13	2	0	100.00	0.52	0.59	1.11	2.60
UBC 840	4	33	4	0	100.00	0.41	0.18	1.18	6.60
UBC 845	5	28	5	0	100.00	0.32	0.78	2.75	5.60
UBC 848	3	7	3	0	100.00	0.89	0.64	2.29	1.40
UBC 852	2	12	2	0	100.00	0.55	0.55	1.10	2.40
UBC 853	4	31	2	2	50.00	0.18	0.37	0.55	6.20
UBC 855	3	22	2	1	66.67	0.29	0.42	0.71	4.40
UBC 881	2	11	2	0	100.00	0.65	0.52	1.17	2.20
Total	41	273	33	8	Average	0.35	0.42	1.07	4.2

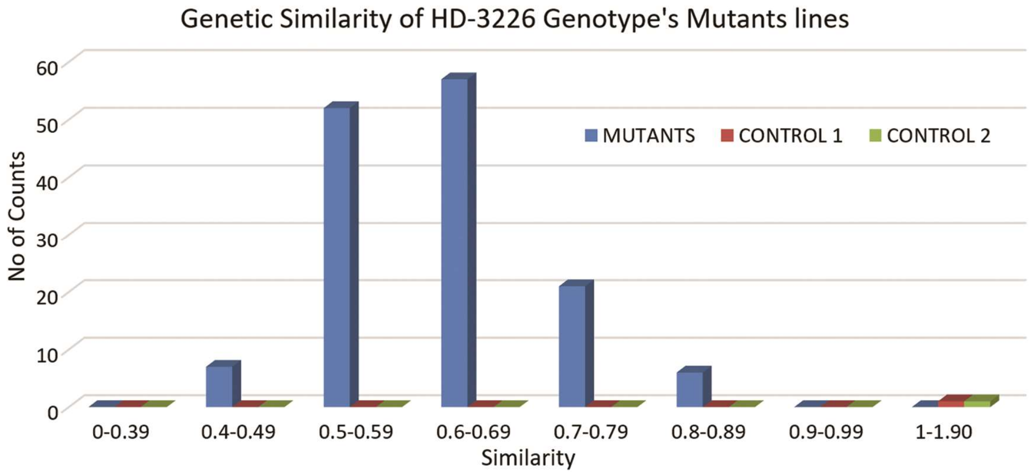


Fig. 3 — Histogram showed the distribution of genetic similarity among mutant lines of HD-3226 genotype

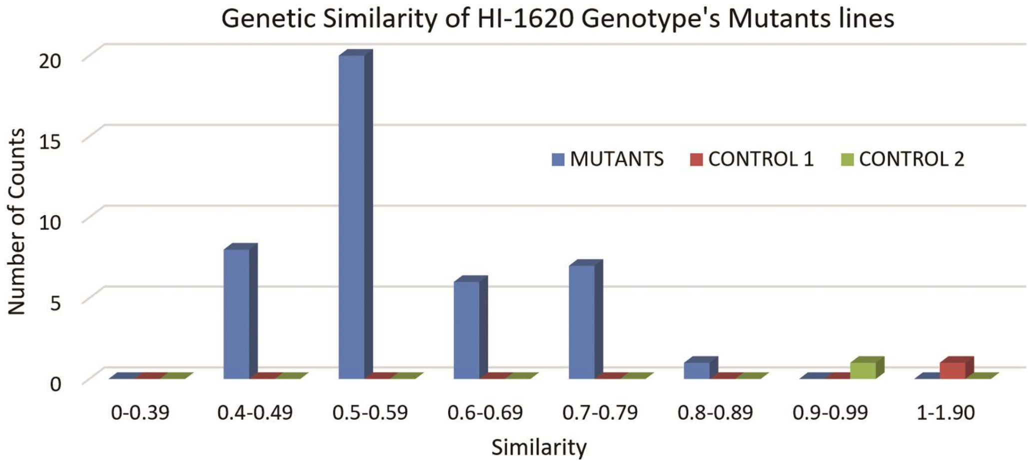


Fig. 4 — Histogram showed the distribution of genetic similarity among mutant lines of HI-1620 genotype

Table 4 — Jaccard's similarity coefficients based on ISSR marker analysis in HD-3226 wheat genotype's mutants

	C1	C2	Mt1	Mt2	Mt3	Mt4	Mt5	Mt6	Mt7	Mt8	Mt9	Mt10	Mt11	Mt12	Mt13	Mt14	Mt15	Mt16
C1	1.000																	
C2	1.000	1.000																
Mt1	0.690	0.690	1.000															
Mt2	0.666	0.667	0.595	1.000														
Mt3	0.761	0.761	0.690	0.667	1.000													
Mt4	0.690	0.690	0.714	0.738	0.785	1.000												
Mt5	0.619	0.619	0.500	0.619	0.761	0.690	1.000											
Mt6	0.547	0.547	0.667	0.452	0.452	0.571	0.404	1.000										
Mt7	0.428	0.428	0.595	0.476	0.476	0.547	0.476	0.547	1.000									
Mt8	0.595	0.595	0.619	0.690	0.547	0.619	0.595	0.476	0.690	1.000								
Mt9	0.547	0.547	0.761	0.500	0.642	0.714	0.547	0.619	0.785	0.619	1.000							
Mt10	0.547	0.547	0.714	0.595	0.547	0.619	0.452	0.619	0.500	0.667	0.667	1.000						
Mt11	0.667	0.667	0.595	0.667	0.809	0.738	0.667	0.500	0.571	0.547	0.738	0.547	1.000					
Mt12	0.642	0.642	0.619	0.642	0.690	0.571	0.500	0.523	0.500	0.619	0.667	0.667	0.690	1.000				
Mt13	0.523	0.523	0.642	0.571	0.571	0.642	0.523	0.500	0.761	0.690	0.738	0.500	0.667	0.547	1.000			
Mt14	0.667	0.667	0.642	0.571	0.857	0.690	0.667	0.500	0.523	0.500	0.642	0.500	0.714	0.595	0.571	1.000		
Mt15	0.714	0.714	0.833	0.571	0.761	0.833	0.667	0.642	0.619	0.642	0.833	0.642	0.714	0.595	0.667	0.714	1.000	
Mt16	0.690	0.690	0.571	0.690	0.880	0.714	0.690	0.523	0.452	0.571	0.571	0.571	0.738	0.761	0.500	0.785	0.690	1.000

Genotype HD- 3226:-C1-Control with water, C2- Control with 15% PEG, Mt1-0.25% EMS (water, P-21), Mt2-0.25% EMS (water, P-10), Mt3-0.25% EMS (15% PEG, P-19), Mt4-0.5% EMS (water, P-49), Mt5-0.5% EMS (15% PEG, P-41), Mt6-0.75% EMS (15% PEG, P-63), Mt7-0.75% EMS (15% PEG, P-67), Mt8-1.0% EMS (15% PEG, P-19), Mt9-1.0% EMS (15% PEG, P-25), Mt10-0.02% SA (water, P-4), Mt11-0.02% SA (15% PEG, P-28), Mt12-0.02% SA (15% PEG, P-55), Mt13-0.04% SA (water, P-7), Mt14-0.04% SA (15% PEG, P-2), Mt15-0.04% SA (15% PEG, P-43) and Mt16-0.04% SA (15% PEG, P-36)

Table 5 — Jaccard's similarity coefficients based on ISSR marker analysis in HI-1620 wheat genotype's mutants

	C1	C2	Mt1	Mt2	Mt3	Mt4	Mt5	Mt6	Mt7	Mt8
C1	1.000									
C2	0.970	1.000								
Mt1	0.676	0.676	1.000							
Mt2	0.764	0.764	0.705	1.000						
Mt3	0.617	0.617	0.558	0.588	1.000					
Mt4	0.500	0.500	0.617	0.529	0.500	1.000				
Mt5	0.500	0.500	0.558	0.411	0.441	0.441	1.000			
Mt6	0.735	0.735	0.617	0.823	0.558	0.500	0.441	1.000		
Mt7	0.470	0.470	0.588	0.441	0.470	0.529	0.764	0.570	1.000	
Mt8	0.529	0.529	0.529	0.617	0.705	0.529	0.588	0.588	0.617	1.000

Genotype HI-1620:-C1- Control with water, C2- Control with 15% PEG, Mt1-0.25% EMS (water, P-11), Mt2- 0.25% EMS (15% PEG, P-4), Mt3- 0.25% EMS (15% PEG, P-45), Mt4- 1.0% EMS (15% PEG, P-21), Mt5- 0.02% SA (15% PEG, P-28), Mt6- 0.02% SA (15% PEG, P-4), Mt7- 0.08% SA (15% PEG, P-1) and Mt8- 0.08% SA (15% PEG, P-2)

mutant line (Mt6). Cluster II contains two mutant lines (Mt13 and Mt7). Cluster III divided into two sub groups (one group contains two mutant lines- Mt12 and Mt10 while another group contains two mutant lines- Mt8 and Mt2), cluster IV contains one mutant line (Mt5) and cluster V divided into two sub groups (one group contains four- Mt4, Mt9, Mt15 and Mt1 while another group contain four- Mt11, Mt14, Mt16 and Mt13) were clustered in completely distinct branches. The genetic distance ranged between 0.54-1.00 based on allelic banding pattern of thirteen primers. Highest genetic distance was found between C1, C2-wild type HD-3226 and Mt6-0.75% EMS (15% PEG, P-63) (Fig. 5). In HI-1620 genotype, dendrogram contained four clusters at 0.65 coefficients, cluster I, II, III and IV. Cluster I contains two mutant lines (Mt7 and Mt5). Cluster II contains one mutant line (Mt4). Cluster III contains two mutant lines (Mt8 and Mt3) and cluster IV divided into two sub groups (one group contain- Mt1 while another group contains two mutant lines- Mt6 and Mt2) were clustered in completely distinct

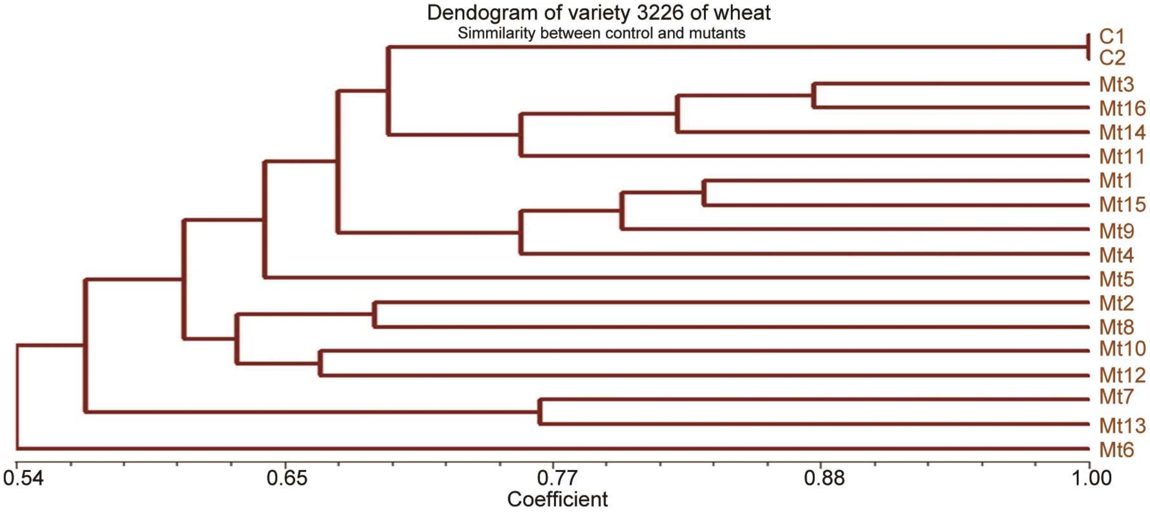


Fig. 5 — Dendrogram of eighteen normal and mutant plants of HD-3226 wheat genotype wheat by UPGMA cluster analysis of genetic similarity based on ISSR data.

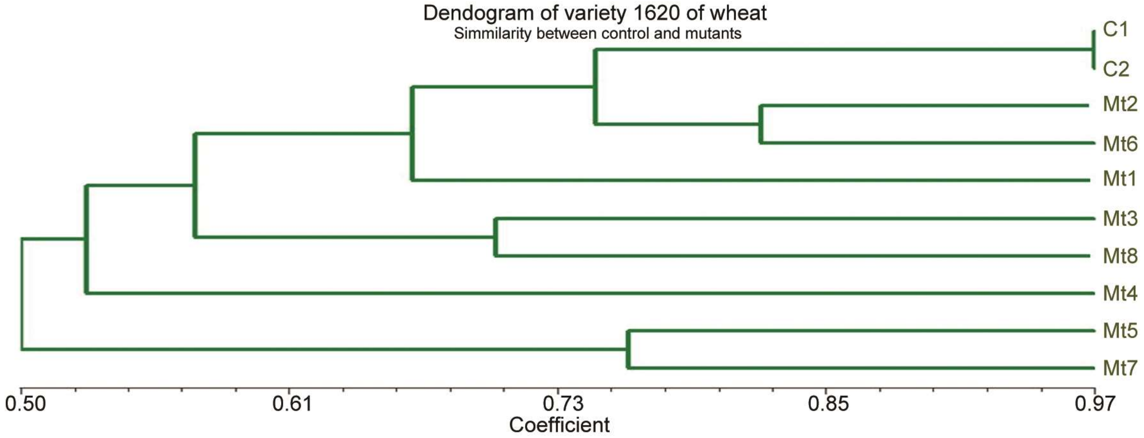


Fig. 6 — Dendrogram of ten normal and mutant plants of HI-1620 wheat genotype by UPGMA cluster analysis of genetic similarity based on ISSR data

branches (Fig. 6). Genetic distance ranged between 0.50-0.97 based on allelic banding pattern of thirteen primers. Highest genetic distance was found between C1,C2-wild type of HI-1620 genotype with Mt5-0.02% SA (15% PEG, P-28) and Mt7-0.08% SA (15% PEG, P-1) followed by Mt4-1.0% EMS (15% PEG, P-21). Surveying loci with high polymorphism is crucial for studying the relationships among wheat genotypes. Although ISSR markers show lower polymorphism, they are cost-effective, valuable for generating molecular markers, and transferable across related genera.

Discussion

Induced mutations can accelerate breeding programs by creating variability through selection and facilitating the multiplication of desirable genotypes[28]. Mutation breeding can be utilized in plant breeding and functional genomics to develop crop varieties with better tolerance or resistance to abiotic stresses[29]. ISSR markers are advantageous because it does not need prior genomic information, as it generates polymorphism patterns by amplifying random loci through PCR. In this study, fifteen drought-specific ISSR markers were used to assess genomic variations in mutant lines of wheat genotypes HI-1620 and HD-3226 treated with different EMS and SA concentrations. High PIC values indicated the primers' effectiveness in distinguishing closely related genotypes. Phenotypic and genotypic data were compared to identify suitable mutant lines for moisture stress tolerance. Out of the 15 ISSR primers tested, 13 produced polymorphic, scorable, and reproducible bands, while two primers failed to amplify in any of the wheat genotypes. Among these, twelve primers-UBC-810, UBC-814, UBC-815, UBC-823, UBC-834, UBC-840, UBC-845, UBC-848, UBC-852, UBC-853, UBC-855, and UBC-881 generated polymorphic bands. Additionally, primer UBC-826 exhibited monomorphic banding patterns across both wheat genotypes and all 24 mutant lines. In HD-3226 genotype mutant 0.25% EMS (water, P-21) showed highest nine variation sites with markers UBC-810, UBC-815, UBC-840, UBC-845, UBC-848, UBC-852, UBC-853, UBC-855 and UBC-881 while mutant 0.08% SA (15% PEG, P-1) showed highest eight variation sites with markers UBC-810, UBC-814, UBC-815, UBC-823, UBC-840, UBC-845, UBC-853 and UBC-881 in genotype HI-1620 (Table 6). In the HD-3226 genotype, PIC values ranged from 0.21 to 0.81, with marker index values from 0.59 to 3.95. Nine primers showed 100% polymorphism, while UBC 823 showed 50%. Resolving power ranged from 2.33 to 9.56. In the HI-1620 genotype, PIC values ranged from 0.00 to 0.89, and marker index values ranged from 0.30 to 2.75. Eight primers showed 100% polymorphism, while UBC 853 showed 50%. Resolving power ranged from 1.40 to 7.00, demonstrating the primers' ability to differentiate closely related wheat mutant lines. Previously, George *et al.* used ten ISSR primers to evaluate polymorphism rate for ranged 62.5% to 100%, with an average of 83.3% for all primers[30]. Moreover, Jabari *et al.* discovered genetic diversity in *Aegilops* and *Triticum* species to provide an ideal gene pool for discovering genes useful for wheat breeding[31]. Shaban *et al.* observed polymorphism among ten wheat genotypes using seven ISSR primers under normal and drought conditions[32]. The genetic diversity between mutant lines and control-wild type obtained 122 total fragments of DNA from twenty-four ISSR primers and 82 (67.21%) were polymorphic as reported by Sen *et al*[5]. Additionally, Xi *et al.,* identified mutations in nine mutant lines as compared with control (WT) plantlet through ISSR loci banding pattern[33]. Similar, studies of genetic diversity in wheat using ISSR were also reported by Golea *et al.*[34], Ateş-Sönmezoğlu *et al.*[35] and Hajiyev *et al.*[36]. In present study, all concentrations of EMS and SA are capable to create mutation at markers site of ISSR in the both HD-3226 and HI-1620 genotype.

Table 6 — Selected M_3 twenty four mutant plants shows variations in their banding patterns of genomic DNA using ISSR markers

S. No.	Mutants	Variation at marker sites
1	EMS-0.25% HD-3226 WATER (P-21)	UBC 810,UBC 815,UBC 840,UBC 845,UBC 848,UBC 852,UBC 853,UBC 855 and UBC 881
2	EMS-0.25% HD-3226 WATER (P-10)	UBC 834,UBC 840 and UBC 881
3	EMS-0.25% HD-3226 15% PEG (P-19)	UBC 840,UBC 845,UBC 848 and UBC 852
4	EMS-0.5% HD-3226 WATER (P-49)	UBC 814,UBC 834,UBC 845,UBC 848,UBC 852 and UBC 881
5	EMS-0.5% HD-3226 15% PEG (P-41)	UBC 810,UBC 823,UBC 845,UBC 848,UBC 852,UBC 853 and UBC 881
6	EMS-0.75% HD-3226 15% PEG (P-63)	UBC 810, UBC 814, UBC 815,UBC 840,UBC 848,UBC 852 and UBC 881
7	EMS-0.75% HD-3226 15% PEG (P-67)	UBC 823,UBC 840,UBC 845,UBC 848,UBC 852,UBC 853 and UBC 881
8	EMS-1.0% HD-3226 15% PEG (P-19)	UBC 840,UBC 845,UBC 853 and UBC 881
9	EMS-1.0% HD-3226 15% PEG (P-25)	UBC 840,UBC 845,UBC 848,UBC 852,UBC 853and UBC 881
10	SA- 0.02% HD-3226 WATER (P-4)	UBC 823,UBC 840,UBC 845,UBC 853,UBC 855 and UBC 881
11	SA- 0.02% HD-3226 15% PEG (P-28)	UBC 823,UBC 845,UBC 848,UBC 852 and UBC 853
12	SA- 0.02% HD-3226 15% PEG (P-55)	UBC 815,UBC 823,UBC 845,UBC 853,UBC 855 and UBC 881
13	SA- 0.04% HD- 3226 WATER (P-7)	UBC 810,UBC 845,UBC 848,UBC 852,UBC 853,UBC 855 and UBC 881
14	SA- 0.04% HD- 3226 15% PEG (P-2)	UBC 810,UBC 823,UBC 845,UBC 848,UBC 852 and UBC 853
15	SA- 0.04% HD-3226 15% PEG (P-43)	UBC 810,UBC 823, UBC 840,UBC 845,UBC 848,UBC 852,UBC 853and UBC 881
16	SA- 0.04% HD-3226 15% PEG (P-36)	UBC 814, UBC 815, UBC 840, UBC 845, UBC 848, UBC 852, UBC 853 and UBC 855
17	EMS-0.25% HI- 1620 WATER (P-11)	UBC 810,UBC 815, UBC 848,UBC 852,UBC 855 and UBC 881
18	EMS-0.25% HI- 1620 15% PEG (P-4)	UBC 810,UBC 845,UBC 853 and UBC 855
19	EMS-0.25% HI- 1620 15% PEG (P-45)	UBC 810, UBC 815, UBC 823, UBC 845, UBC 848, UBC 852, UBC 855 and UBC 881
20	EMS-1.0% HI- 1620 15% PEG (P-21)	UBC 840,UBC 845,UBC 848,UBC 852,UBC 855 and UBC 881
21	SA- 0.02% HI- 1620 15% PEG (P-28)	UBC 810,UBC 815, UBC 840,UBC 845,UBC 853,UBC 855 and UBC 881
22	SA- 0.02% HI- 1620 15% PEG (P-4)	UBC 810,UBC 823,UBC 845 and UBC 853
23	SA- 0.08% HI- 1620 15% PEG(P-1)	UBC 810,UBC 814, UBC 815,UBC 823, UBC 840,UBC 845,UBC 853 and UBC 881
24	SA- 0.08% HI- 1620 15% PEG (P-2)	UBC 810, UBC 823, UBC 840,UBC 845,UBC 848,UBC 852 and UBC 881

Jaccard's coefficient method was used for estimation of pairwise genetic similarity among M_3 mutant lines of two wheat genotypes. In HD-3226 genotype, mutant line Mt5-0.5% EMS (15% PEG, P-41) and Mt6-0.75% EMS (15% PEG, P-63) was found to closely dissimilar (0.404) to each other. The highest genetic similarity (0.880) was detected between mutant lines Mt16-0.04% SA (15% PEG, P-36) and Mt3-0.25% EMS (15% PEG, P-19). On the other hand HI-1620 genotype, mutant lines Mt5-0.02% SA (15% PEG, P-28) and Mt2-0.25% EMS (15% PEG, P-4) were found to closely dissimilar (0.411) to each other. The highest genetic similarity (0.823) was detected between mutant lines Mt6-0.02% SA (15% PEG, P-4) and Mt2-0.25% EMS (15% PEG, P-4). Further, genetic similarity matrix was analyzed using UPGMA. Dendrogram was generated on the basis of absence and presence of different alleles in mutant lines generated by 13 ISSR markers. In HD-3226 genotype, dendrogram derived from this analysis was depicted as it contain five clusters at 0.65 coefficient, cluster I, II, III, IV and V. According to allelic banding pattern of thirteen primers, genetic distance varied from 0.54 to 1.00. Highest genetic distance was found between C1, C2-wild type of HD-3226 genotype and Mt6-0.75% EMS (15% PEG, P-63). In HI-1620 genotype, dendrogram contained four clusters at 0.65 coefficient, cluster I, II, III and IV. According to allelic banding pattern of thirteen primers, genetic distance varied from 0.50 to 0.97. Highest genetic distance was found between C1, C2- wild type of HI-1620 with Mt5-0.02% SA (15% PEG, P-28) and Mt7-0.08% SA (15% PEG, P-1) followed by Mt4-1.0% EMS (15% PEG, P-21). Similarly, genetic affinity index and cluster analysis revealed the closest genotypes of YBFS48 (var. albidum) and var. YBFS43 (barbarossa)[36]. Moreover, Polido *et al.* observed that average genetic similarity was 0.57, with a range of 0.29 to 0.93 and dendrogram had four groups, and they reported that genotypes LD-111202 and LD-111203 had a significant degree of genetic similarity with one another[37]. Additionally, Singh *et al.,* reported that PIC value ranged 0.14 to 0.467 with an average of 0.287 and Mantel test revealed a significant connection of microsatellite genetic distance (r= 0.77557)[38].

Conclusion

In present study, twelve primers were showed polymorphic in twenty four mutant lines along with their controls-wild type in two wheat genotypes. Out of twenty four mutants, total sixteen mutants were generated through EMS (nine) and SA (seven) of HD-3226 genotype and remaining eight mutants were generated with treatment of EMS (four) and SA (four) of the HI-1620 genotype. The results showed HI-1620 genotype may be more tolerant against EMS and SA as compared to HD-3226 genotype this may be due to a better DNA repair mechanism or protection of DNA. This study suggests that ISSR markers are effective for analysing genetic variability in closely related genotypes[39,40]. All molecular markers exhibited a resolving power greater than 1.0, indicating their high efficiency in detecting polymorphisms and distinguishing between genotypes. This makes them highly suitable for applications such as genetic diversity assessment, linkage mapping, and marker-assisted selection. A high PIC value suggests that the primers are highly informative and effective in detecting genetic variation among mutant lines, making them valuable for future research in genetic resource classification and conservation. Cluster analysis highlights that the distribution and grouping of genetic diversity are crucial for categorizing samples into clusters suitable for specific regions. Additionally, ISSR-based genetic similarity data assist researchers in selecting superior mutant plantlets for crop improvement or in developing new cultivars through mutagenesis. EMS and SA have been effectively used to induce mutations in wheat seeds. The identified M_3 mutant lines can serve as important material for reverse genetics and functional genomics studies.

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Conflicts of interest

All authors declared no conflict of interest.

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