Генетика и селекция растений
Genetics and plant breeding

DOI: 10.22363/2312-797X-2026-21-1-41-54
EDN DZOGID
UDC 631.52:631.671.3:633.111.1

Research article / Научная статья

Identification of drought-tolerant bread wheat *Triticum aestivum L.* genetic resources using molecular markers

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Abstract. To mitigate climate change and support the global agricultural economy, plant breeding and improvement programmes have received significant attention to obtain genetic resources rich in tolerant genes. In our study, twenty ISSR primers were used to evaluate genetic variation for 23 genotypes. SSR markers and the Sanger sequencing method were used to detect drought-tolerant genotypes and identify target gene loci. ISSR-PCR results showed a total of 820 DNA bands, of which 172 bands were polymorphic (117 non-unique bands and 55 unique bands) with a polymorphism percentage of 88.6. In the similarity matrix and dendrogram, genotypes were divided into clusters according to the genetic origins of the parents. These results demonstrated that ISSR markers are a valuable method for determining genetic variation, and identification of wheat genetic origins. Using primers SSR-PCR (*Malek 1, Malek 2*), genotypes (AB, NF, CL, UD, SA, TB) were detected as having drought tolerance genes. DNA sequencing by Sanger sequencing method of the genotypes, genes (*DRF1, NAC20L*) were identified in the wild parent (*Triticum turgidum L*), which is considered the source of the tolerant genes. Sample sequences and genotypes were recorded in the Gene Bank and The *NCBI* Bankit platforms. DNA sequencing technology has proven its effectiveness in confirming field results and identifying targeted genetic sites in the wheat genome.

Keywords: drought stress, ISSR, SSR, DNA sequencing, Sanger sequencing

Authors' contribution: Walli M.H., Zargar M. — concept development, analysis and interpretation of results, scientific writing; Duksi F., Al-jubouri Z., Alhasnawi A. — data collection and processing, approval of the final version of the manuscript. All authors reviewed the final version of the manuscript and approved it.

Conflict of interests. The authors declare no conflict of interests.

Article history: received 2 July 2025; accepted 14 January 2026.

For citation: Walli MH, Duksi F, Al-jubouri Z, Zargar M, Alhasnawi A. Identification of drought-tolerant bread wheat *Triticum aestivum* L. genetic resources using molecular markers. *RUDN Journal of Agronomy and Animal Industries*. 2026;21(1):41–54. doi: 10.22363/2312-797X-2026-21-1-41-54 EDN DZOGID

Идентификация генетических ресурсов засухоустойчивости продовольственной пшеницы *Triticum aestivum L.* с использованием молекулярных маркеров

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Аннотация. Для смягчения последствий изменения климата и поддержки мировой аграрной экономики значительное внимание уделяется программам селекции и улучшения растений с целью получения генетических ресурсов, богатых генами засухоустойчивости. Оценку генетической изменчивости 23 генотипов проводили с использованием двадцати ISSR-праймеров. Выявляли засухоустойчивые генотипы и осуществляли идентификацию целевых локусов генов примененеим SSR-маркеров и метода секвенирования по Сэнгеру. Результаты ISSR-ПЦР показали в общей сложности 820 полос ДНК, из которых 172 полосы были полиморфными (117 неуникальных полос и 55 уникальных полос) с процентом полиморфизма — 88,6. В матрице сходства и дендрограмме генотипы были разделены на кластеры в соответствии с генетическим происхождением материнских форм. Полученные результаты продемонстрировали, что ISSR-маркеры являются ценным методом определения генетической изменчивости и идентификации генетического происхождения пшеницы. С помощью праймеров SSR-PCR Malek 1, Malek 2 выявили гены засухоустойчивости у генотипов AB, NF, CL, UD, SA, TB. Секвенирование ДНК генотипов методом Сэнгера позволило идентифицировать гены DRF1 и NAC20L у дикого родительского растения (*Triticum turgidum L.*), которое считается источником генов засухоустойчивости. Образцы последовательностей и генотипов зарегистрировали в Gene Bank и на платформах NCBI Bankit. Технология секвенирования ДНК доказала свою эффективность в подтверждении результатов полевых исследований и идентификации целевых генетических участков в геноме пшеницы.

Ключевые слова: засуха, ISSR, SSR, секвенирование ДНК, генетика, секвенирование по Сэнгеру

Вклад авторов: Валли М.Х., Заргар М. — разработка концепции, анализ и интерпретация данных, подготовка и редактирование текста; Дукси Ф., Аль-джабори З., Альхаснави А. — сбор и обработка материала, согласование окончательного варианта статьи. Все авторы одобрили окончательную версию статьи.

Заявления о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

История статьи: поступила в редакцию 2 июля 2025 г., принята к публикации 14 января 2026 г.

Для цитирования: *Walli M.H., Duksi F., Al-jubouri Z., Zargar M., Alhasnawi A.* Identification of drought-tolerant bread wheat *Triticum aestivum* L. genetic resources using molecular markers // Вестник Российского университета дружбы народов. Серия: Агрономия и животноводство. 2026. Т. 21. № 1. С. 41–54. doi: 10.22363/2312-797X-2026-21-1-41-54 EDN: DZOGID

Introduction

Global climate change is causing significant challenges for countries like Iraq, affecting over 70% of its irrigated areas [1]. The issue of rising temperatures, evaporation, and river desiccation, combined with dam construction and saline groundwater irrigation, poses a threat to food security [1, 2]. Wheat (*Triticum aestivum*) is among the most important food crops worldwide in production and use as food. The Food and Agriculture Organization (FAO) reports that wheat production averaged 765140714.8 tons from 2016 to 2016–2022, making it the third strategic crop globally after maize and sugarcane. The amount of wheat production in Asia reached 335,995,076.7 tonnes[1]. Iraq is one of the most important centres for wheat production and breeding, contributing more than 20% of the world's food needs [3]. This cereal crop is the top choice among all cereal crops and is highly valued economically, making it a crucial strategic food crop. Wheat production in Iraq totalled roughly 3,683,604,429 tonnes, cultivated over an area of 1,334,193 hectares from 2016 to 2022. Increasing world population, coupled with abiotic stresses and global warming, has led to a decline in agricultural areas [4]. The Tigris and Euphrates rivers' declining water supply has worsened drought and water salinity, necessitating vertical agricultural expansion, requiring plant breeders to develop tolerant varieties [5]. Inter Simple Sequence Repeat ISSR markers are crucial in identifying genetic variations in wheat plants [6]. ISSR markers reveal significant genetic variation in wheat germplasms, with high polymorphic loci percentages observed in 80.5% bread wheat and 98.2% durum wheat populations [7, 8]. DNA sequencing methodologies have advanced wheat breeding and enhancement studies by discovering genetic variation linked to specific traits [9]. Utilising modern biotechnological techniques, including DNA markers and gene sequencing, to identify genes in diverse crop species is a dependable approach for locating drought-tolerant genetic resources in contrast with physical and biological features that may be affected by environmental factors.

The objectives of this study were to perform molecular diagnosis of drought-tolerant wheat genotypes and to identify drought tolerance genes through genomic sequencing.

Materials and Methods

Materials: The study included 23 genotypes of bread wheat (*Triticum aestivum L.*), which are listed in Table 1. We collected 18 genotypes from the Gene Bank and accredited research centres affiliated with the Ministry of Agriculture and Higher Education in Iraq, while other genotypes are from the Russian Federation, France, Italy, Spain and Turkey.

[1] FAO. *Food and Agriculture Organization of the United Nations. FAOSTAT*. 2022. https://www.fao.org/faostat/en/#data/HS

Table 1

Genetic sources (genotypes) in the study, origin, sources

N	Genotype	Sample	Pedigree	Origin
1	Baraka	AB	IARI × STD	Iraq (hybridization)
2	Wafia	BW	Attila / 3* Pastor,(CIMMYT, ICARDA)	Imported from France
3	Latifiya	CL	Australian breed × Aras	Iraq (hybridization)
4	Binakal	DB	BISU/3/YAV79/ALOI/ALTARS4/ CD93683.7Y.040M-03 OY-LPAP.B	Imported from Spanish
5	Uruk	EU	Inia 66 (Rad) Irradiation of seeds of Enya 66	Iraq (Irradiation)
6	Sham	FS	W-3018-A/JUPATECO-73	Iraq (Irradiation)
7	Fateh	GF	MixPac × Aras	Iraq (hybridization)
8	Buhuth 10	HB	Abaa 95 × Abaa 99	Iraq (hybridization)
9	Buhuth 158	IB	119-S2/57-S2. Cr7.S2	Iraq (Irradiation)
10	Babul 113	JB	MEXIPAK/R23	Iraq (Irradiation)
11	Al Iraq	KA	Irradiation of Mexipac seeds with full cobalt 60 doses and 10 kilos rad, Max. (Rad)	Iraq (Irradiation)
12	Bwru	LB	H31/Trapf21 / Enesco	Italy
13	Baghdad	MB	MX105–6MVLT40 / BNSN	Iraq (hybridization)
14	Faris	NF	STAR/TR77/773/SLMS	Iraq (Irradiation)
15	Tammuz	OT	Exposing the resulting hybrid (Maxipac x Saber Beek) to radiation	Iraq (hybridization and irradiation)
16	Buhuth	PB	118//S2/57-S2-CR7-S2	Iraq (hybridization)
17	Abaa 95	QA	Veery eer	CIMMYT
18	Abaa 99	RA	Ures/Boww/oowwJup/ Biyiy	CIMMYT
19	Abo ghurayb	SA	Ajeeba × Lian 12 × Mexico24	Iraq (hybridization)
20	Buhuth 22	TB	CMSS96Y03236M-050M-040M-020M-050Sy-020sy-IM-0Y	Iraq (Irradiation)
21	Dujela	UD	8409644HS2–6H	Iraq (Irradiation)
22	Nemchinovka	VN	(Donshchina × Pamyati Fedina) × Moskovskaya 39	Imported from Russia
23	Abo Raghif	WA	Inia 66 / 2 × Mexipak	Imported from Turkey

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

DNA Extraction: Genomic DNA was isolated from 10-day-old fresh leaves of 23 bread wheat genotypes utilising the Favorgen Plant DNA Maxi Kit (FAVORGEN BIOTECH CORP) in accordance with the manufacturer's guidelines [10, 11]. DNA purity and concentration were verified using a NanoDrop spectrometer, and samples were diluted with TE solution to the required concentration.

PCR amplification utilising ISSR primers: In this study, 20 ISSR primers were used to detect genetic variation in wheat cultivars, based on the manufacturer Alpha DNA Canada in Table 2 [10, 12]. The PCR reaction (total volume of 25 µl) contained 2 µl of genomic DNA, 1 µl of ISSR primer, 12.5 µl of Master Mix, and 9.5 µl of nuclease-free water. PCR cycles started with an initial denaturation phase of 3 minutes at 94 °C, succeeded by 32 cycles comprising denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 45–59 °C (as specified by the primers' annealing temperature in Table 2), extension for 1 minute at 72 °C, and concluding with a final extension step of 5 minutes at 72 °C. PCR products were separated by electrophoresis on 1.5% agarose gels in 1×TBE buffer at 125 V for 30 minutes. A 100–1500 bp DNA ladder was used as a size marker. Gels were visualized under UV light (260 nm). For each accession, reproducible ISSR bands were scored by analysing gel images using Gel Analyzer 23.1.1 software to convert band patterns into digital data.

Table 2

Primer sequence (5'–3'), temperature, and length for twenty ISSR markers in this study [12–14]

N	Primers	Sequences 5'-3'	Length (meres)	Annealing, °C
1	ISSR-1	$(AG)_9C$	19	58
2	ISSR-2	$(AC)_8G$	17	52
3	ISSR-3	$(AG)_8T$	17	50
4	ISSR-4	$(TG)_8C$	17	52
5	ISSR-5	$(AC)_8Y$	17	48
6	ISSR-6	$A(CAG)_5$	16	52
7	ISSR-7	$B(CT)_8Y$	18	50
8	ISSR-8	$R(ACA)_5$	16	40
9	ISSR-9	$(CAC)_7G$	22	74
10	ISSR-10	$(CT)_9Y$	19	56
11	ISSR-11	$(CA)_8DT$	18	50
12	ISSR-12	$(CTC)_6G$	19	64
13	ISSR-13	$(GT)_8C$	17	52
14	ISSR-14	$G(CA)_8$	17	52
15	ISSR-15	$(GAA)_7$	21	56
16	ISSR-16	$(GA)_8V$	17	48
17	ISSR-17	$(GT)_8C$	17	52
18	ISSR-18	$(GA)_8G$	17	52
19	ISSR-19	$(GA)_8HC$	18	52
20	ISSR-20	$(GACA)_5$	20	60

Note. B = C, G, T; D = A, G, T; H = A, C, T; R = A, G; V = A, C, G; Y = C, T.

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Detection of polymorphism of genotypes and primers: Numeric data signifies the presence of band 1 and the absence of band 0. Bands exhibiting identical mobility were assessed as equivalent. The Polymorphic Information Content (PIC) values for each primer were calculated by assessing the allele frequency at each locus according to [15, 16] which the marker possesses, and their relative rates. There are two indexes, or measures, usually used for the polymorphism degree evaluation. They are the heterozygosity (H, using the formula

$$PIC = 2fi(1-fi),$$

where fi is the frequency of the amplified allele.

The Effective Multiplex Ratio (EMR) refers to the quantity of polymorphic fragments identified per assay. EMR and Marker Index (MI) for the marker system were computed to assess the efficacy of the marker system [17].

$$EMR = np/n,$$

where np represents the count of polymorphic loci, and n is the total number of loci.

$MI = EMR \times PIC$. The Multiplex Ratio (MR) was calculated by dividing the total number of amplified bands by the total number of assays. The resolving power (Rp), indicating the capacity of the most informative primers to distinguish between genotypes, was evaluated according to [15, 16] using: $Rp = \sum Ib$, where Ib represents band informativeness, defined as $Ib = 1 — [2x(0.5 — p)]$, where p denotes the proportion of clones that possess the band. The resolving power depends on the distribution of identified bands among the sampled genotypes.

The coefficient of genetic similarity (GS) between genotypes was estimated by the Dice coefficient from binary data [17]. Dice formula

$$GSij = 2a/(2a + b + c),$$

where $GSij$ is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The similarity matrix was employed to assess the cluster. The distance between each pair of groups was calculated as the average distance among all pairings inside the two related groups until all groups were interconnected. A dendrogram was used using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) by the between-groups linkage method in SPSS to know the genetic relations between genotypes of wheat [16, 17].

PCR amplification by using SSR (microsatellite) markers: The purpose of the test: Enhancing the morphological results that appeared in the field; detecting genes that are tolerant to drought using molecular markers. Group A, with the genotypes AB, CL, NF, SA, TB and UD, was selected after analysing field experimental data and classifying genotypes according to tolerance to drought levels, with the addition of three of the genotypes, PB, JB and LB, from other groups to demonstrate the accuracy of SSR markers. Selection of the TaDREB3 gene in chromosomal locations of bread wheat that have shown their ability to contribute to tolerance to water stress [18], so primers were made; therefore, new primers (Malek 1, Malek 2) were selected in this study using the NCBI Primer-BLAST tool and Primer3 Plus software. These were used alongside previously known primers (Xgwm130 and Xwmc245) [19, 20] to detect drought-tolerant wheat genotypes.

Genomic DNA of wheat genotypes was subjected to SSR analysis using four primers (Table 3) as genetic markers associated with drought tolerance and approved in several sources in the selective breeding programme [19]. The primers were made by Alpha ADN, S.E.N.C., www.alphaadn.com. A total of 36 samples were analysed in the SSR test, with 9 samples per primer representing the genotypes included in the study. To prepare the template, there are several materials added according to the manufacturer of the Master Max PCR. Samples were numbered before DNA was added. The PCR reaction mixture of 25 µl comprised 12.5 µl of 10X Taq Master Mix with Standard Buffer, 2 µl of template DNA, 9.5 µl of nuclease-free water, 0.5 µl of 10 µM forward primer, and 0.5 µl of 10 µM reverse primer. The reaction mixtures were subjected to an initial heating at 94 °C for 3 minutes, followed by 32 cycles consisting of 94 °C for 30 seconds, 40–55 °C for 30 seconds (depending on the primer used), and 72 °C for 1 minute. A final extension was conducted at 72 °C for 5 minutes. SSR-PCR products were analysed via agarose gel electrophoresis, following these steps: a 3% agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml of 1X TBE. Subsequently, 2 µL of ethidium bromide stain was incorporated into the agarose gel solution. A DNA marker ladder (100–1500 bp) supplied by TRANS-China was loaded into one well. An electric current was applied at 125 V for 25 minutes, followed by 75 V for 1 hour. SSR-PCR products were visualised using ultraviolet (UV) light at 365 nm with a photo imaging system.

Table 3

Names of SSR primers, sequence, length and sources

N	Primer	Primer Sequence	Length (k-mers)	Temperature, ˚C	Source
1	Malek 1	F GGTAGATCGGAAGGACGCTGR CAGGGGGCTCATCACCAAAT	2020	6462	NEW
2	Malek 2	F ATTGCAAGGAGCACATCCGAR TCAGCATCATGGAAGGCAGG	2020	6062	NEW
3	Xgwm130	F AGCTCTGCTTCACGAGGAAGR CTCCTCTTTATATCGCGTCCC	2021	6264	[20]
4	Xwmc245	F GCTCAGATCATCCACCAACTTCR AGATGCTCTGGGAGAGTCCTTA	2222	6666	[21]

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Sequencing Methods: DNA samples of the five genotypes (AB, NF, UD, SA, TB) that showed tolerance to drought in SSR-PCR tests were sent to Alpha ADN Canadian, www.alphaadn.com, by comparing local samples' nucleic acid sequences with retrieved sequences, using Sanger dideoxy sequencing technology. Madison's BioEdit Sequence Alignment Editor Software Version 7.1 was used to analyse the PCR product of a targeted sample. The software was used to compare observed nucleic acid sequences with retrieved sequences, identifying virtual positions and details of PCR fragments. The sequences were numbered in PCR amplicons and corresponding positions within the referring genome, ensuring accurate analysis of the sample. PCR amplicons were used to identify variations in sequenced samples, which were then translated to their corresponding amino acid sequences to assess the impact of these variations on the encoded protein, using the Expasy translate suite. The NCBI Bankit portal was used to submit investigated sequences, which were then analysed and provided as nucleic acid sequences to GenBank for unique accession numbers.

Results and Discussion

Molecular characterisation of wheat genotypes by ISSR-PCR markers. In the PCR-ISSR investigation of the genotypes presented in Table 4, twenty ISSR primers were utilised to amplify a total of 820 fragments, yielding an average of 41 bands per primer MR. The electrophoresis of primers ISSR-8 and ISSR-20 did not demonstrate the existence of fragments, suggesting the absence of bands. In these pieces, 37.6 bands were identified as polymorphic. The number of polymorphic bands produced by ISSR primers ranged from 18 (ISSR-7, ISSR-6) to 71 (ISSR-3, ISSR-18). The percentage of polymorphism between genotypes and lines varied from 37% (ISSR-15) to 100% (ISSR-3, ISSR-13, ISSR-18), yielding an average polymorphism of 68% per primer. Mb comprises 69 bands in ISSR-1, ISSR-15, and ISSR-19. The number of unique bands (Ub) was 55 bands with an average of 2.8 per primer. Primer ISSR-15 detected the highest number of unique bands (7 bands). The highest Marker Index (MI) value was observed for the ISSR-1 primer (11.34) with an average of 5. The highest percentage of Resolving Power (RP) was found for the ISSR-3 and ISSR-18 primers (9.45). Regarding Polymorphic Information Content (PIC), the highest value was recorded for the ISSR-11 primer (0.29) with an average of 0.2. The average molecular weight (MW) of the primers ranged from 184.45 to 2042.3, with the highest MW observed for the ISSR-1 primer (3388). The results of PCR-ISSR analysis showed that most of the primers showed the ability to distinguish between the genotypes in the experiment; the largest number of bands for the KA genotype was 45, with an average of 2.25 for all primers. As for the least number of bands that appeared in the WA genotype, it reached 23 bands, and the average was 2.25.

Table 4

Distinct characteristics of ISSR primers included in the study: primer names, a Total of bands (Tb), Polymorphic bands (Pb), polymorphism (P%), Monomorphic bands (Mb), number of Unique bands (Ub), Non-Unique bands (Non-Ub), Marker Index (MI), Resolving Power (RP), Polymorphic Information Content (PIC), Molecular Weight (MW)

Primers	Mb	Ub	Non-Ub	Pb	Tb	P%	EMR	PIC	RP	MI	MW(bp)
ISSR-1	1	2	5	7	8	87.5	612.5	0.15	1.39	92.0	25–3388
ISSR-2	0	3	4	7	7	100	700	0.23	1.91	158.8	424–1315
ISSR-3	0	5	8	13	13	100	1300	0.20	3.30	253.9	302–2877
ISSR-4	0	2	5	7	7	100	700	0.23	2.09	158.8	145–2457

Ending tabl. 4

Primers	Mb	Ub	Non-Ub	Pb	Tb	P%	EMR	PIC	RP	MI	MW(bp)
ISSR-5	0	4	7	11	11	100	1100	0.23	3.74	257.8	77–3004
ISSR-6	0	1	5	6	6	100	600	0.22	1.65	132.3	153–1603
ISSR-7	0	2	2	4	4	100	400	0.27	1.57	106.6	75–1814
ISSR-8	0	0	0	0	0	0	0	0	0	0	0
ISSR-9	0	3	4	7	7	100	700	0.21	2.96	148.7	700–2729
ISSR-10	0	2	7	9	9	100	900	0.25	2.78	222.3	605–2872
ISSR-11	0	5	9	14	14	100	1400	**0.29**	1.74	**410.2**	268–1622
ISSR-12	0	2	2	4	4	100	400	0.19	0.87	74.1	78–313
ISSR-13	0	3	8	11	11	100	1100	0.21	2.96	235.2	2–1740
ISSR-14	0	4	11	15	15	100	1500	0.21	4.17	319.8	187–3091
ISSR-15	1	7	6	13	14	92.9	1207.1	0.15	2.52	181.9	52–2105
ISSR-16	0	2	4	6	6	100	600	0.24	1.91	142.9	88–2473
ISSR-17	0	2	13	15	15	100	1500	0.21	2.43	315.3	13–2898
ISSR-18	0	4	9	13	13	100	1300	0.25	4.70	330.4	74–1996
ISSR-19	1	2	8	10	11	90.9	909.1	0.18	2.43	166.9	189–2549
ISSR-20	0	0	0	0	0	0	0	0	0	0	0
SUM	3	55	117	172	175	1771.3	16928.7	3.9	45.1	3708.1	3689–40846
MEAN	0.15	2.75	5.85	8.6	8.75	88.6	846.4	0.2	2.3	185.4	184.45–2042.3

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Dendrogram analysis for 23 genotypes based on ISSR-PCR data. Genetic similarity among the 23 genotypes was estimated from the matrix data (Table 5). The Genetic Similarity (GS) values derived from ISSR data ranged from 0.21 (WA vs. CL) to 0.75 (MB vs. RA). The average GS value across all genotypes was 0.48 for the ISSR marker. This indicates that the average distance between genetic structures is 0.52.

Dendrogram in Figure 1 shows the presence of two main groups. The first group (14) included the WA genotype with genetic origins from Turkey. It can be noted that the genetic origins of this variety are far from the genetic groups by up to 0.79. The second group was divided into subgroups. Group 3 included the BW variety with genetic origins from France, and the genetic distance from groups 1 and 2 within the group was about 0.63. Group 1 genotype AB is unique in genetic origins and morphological characteristics from the other groups and is considered the closest to group 2 for genotypes DB and EU. In group 8 most of the genotypes were similar in terms of the genetic origins of the parents in Table 1. The similarity between the group 8 and group 9 genotype VN imported from the Russian Federation is 0.57. The genotypes RA and QA that go back to the same genetic sources (CIMMYT) were linked, as well as RA, which is considered one of the parents in the hybridisation of the variety HB, while the closest similarity between the genotypes MB and RA was 0.75. Group 10 includes the genotypes JB and OT resulting from mutation by radiation descending from the genetic origin of one of the parents (MEXIPAK) in Table 1.

Table 5

Genetic similarity matrix (Dice similarity coefficient) among 23 wheat genotypes for Molecular characterization

	AB	BW	CL	DB	EU	FS	GF	HB	IB	JB	KA	LB	MB	NF	OT	PB	QA	RA	SA	TB	UD	VN	WA
AB	1																						
BW	0.37	1																					
CL	0.32	0.4	1																				
DB	0.47	0.40	0.41	1																			
EU	0.47	0.42	0.40	0.52	1																		
FS	0.30	0.40	0.41	0.35	0.4	1																	
GF	0.26	0.44	0.51	0.44	0.49	0.47	1																
HB	0.35	0.29	0.47	0.46	0.38	0.55	0.51	1															
IB	0.31	0.26	0.51	0.39	0.33	0.44	0.54	0.62	1														
JB	0.29	0.27	0.41	0.46	0.48	0.54	0.45	0.57	0.53	1													
KA	0.33	0.30	0.41	0.45	0.49	0.38	0.49	0.46	0.39	0.45	1												
LB	0.30	0.26	0.41	0.36	0.30	0.58	0.39	0.56	0.51	0.47	0.36	1											
MB	0.37	0.33	0.46	0.40	0.42	0.51	0.53	0.67	0.65	0.51	0.48	0.55	1										
NF	0.32	0.36	0.46	0.45	0.45	0.54	0.59	0.64	0.50	0.57	0.55	0.49	0.58	1									
OT	0.35	0.39	0.37	0.54	0.56	0.50	0.59	0.58	0.54	0.62	0.53	0.43	0.55	0.61	1								
PB	0.26	0.24	0.30	0.29	0.26	0.39	0.38	0.46	0.43	0.43	0.32	0.51	0.54	0.42	0.44	1							
QA	0.31	0.29	0.43	0.39	0.44	0.55	0.47	0.62	0.56	0.54	0.47	0.46	0.65	0.65	0.53	0.57	1						
RA	0.28	0.36	0.43	0.40	0.48	0.46	0.54	0.66	0.60	0.58	0.51	0.44	0.75	0.62	0.68	0.45	0.67	1					
SA	0.41	0.41	0.45	0.47	0.46	0.56	0.51	0.56	0.43	0.50	0.49	0.42	0.53	0.62	0.56	0.41	0.59	0.57	1				
TB	0.40	0.45	0.40	0.42	0.49	0.48	0.62	0.51	0.44	0.50	0.58	0.41	0.53	0.58	0.58	0.42	0.53	0.56	0.59	1			
UD	0.26	0.35	0.38	0.39	0.40	0.56	0.53	0.55	0.47	0.56	0.50	0.39	0.51	0.57	0.62	0.40	0.60	0.52	0.50	0.67	1		
VN	0.27	0.25	0.42	0.37	0.34	0.55	0.41	0.64	0.57	0.53	0.44	0.45	0.58	0.56	0.54	0.49	0.56	0.57	0.57	0.49	0.55	1	
WA	0.26	0.28	0.21	0.27	0.32	0.32	0.23	0.39	0.37	0.36	0.33	0.30	0.38	0.45	0.41	0.34	0.44	0.42	0.43	0.38	0.39	0.47	1

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Molecular diagnosis of drought-tolerant genotypes by SSR markers. The PCR-SSR marker analysis was conducted. Gel electrophoresis of the amplified products revealed bands indicating drought tolerance in the tested genotypes. For primer Malek 1, gel electrophoresis revealed the presence of radioactive bands corresponding to the genotypes CL, NF, SA, UD, and TB (Figure 2). For primer Malek 2, bands were observed for genotypes TB, UD, SA, and NF (Figure 2). These radioactive bands indicate the interaction of the primers with the DNA genome of the candidate samples. As for the genotypes (PB, JB, LB), they did not appear in either primer, which confirms the validity of the morphological and physiological results in the field. Primers Xgwm130 and Xwmc245 did not show any binding or interaction with the samples due to the genetic differences between the wheat varieties. This finding aligns with the research objective of identifying new strains tolerant to environmental conditions, which could facilitate wheat breeding and improvement programs aimed at developing new genetic patterns with high productivity under harsh environmental conditions. Based on these results, the genotypes NF, UD, SA, TB, AB and CL were selected for subsequent sequencing and genetic mapping studies.

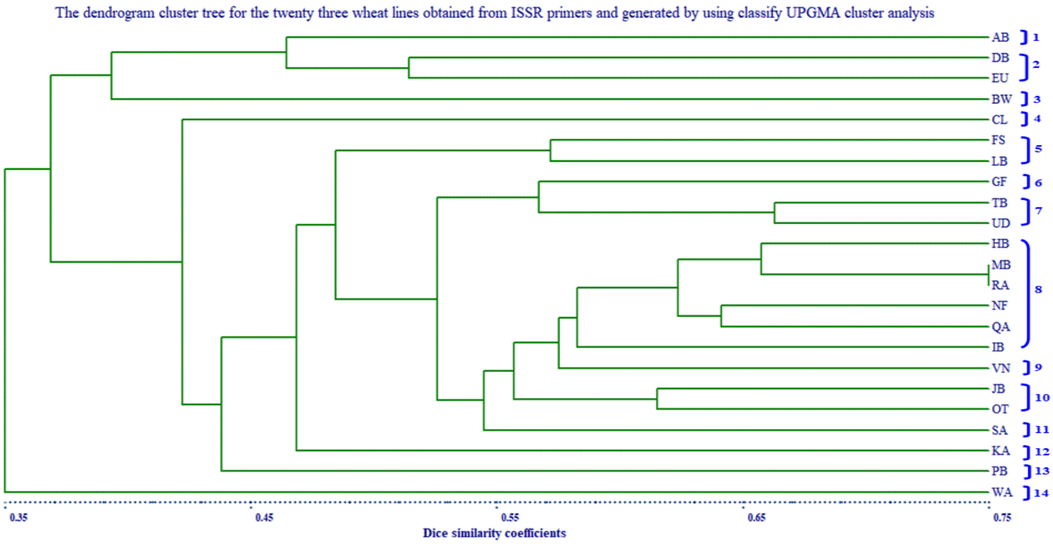

Fig. 1. The dendrogram generated by using UPGMA cluster analysis according to the Dice similarity coefficient obtained from ISSR primers for the twenty-three bread wheat genotypes

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

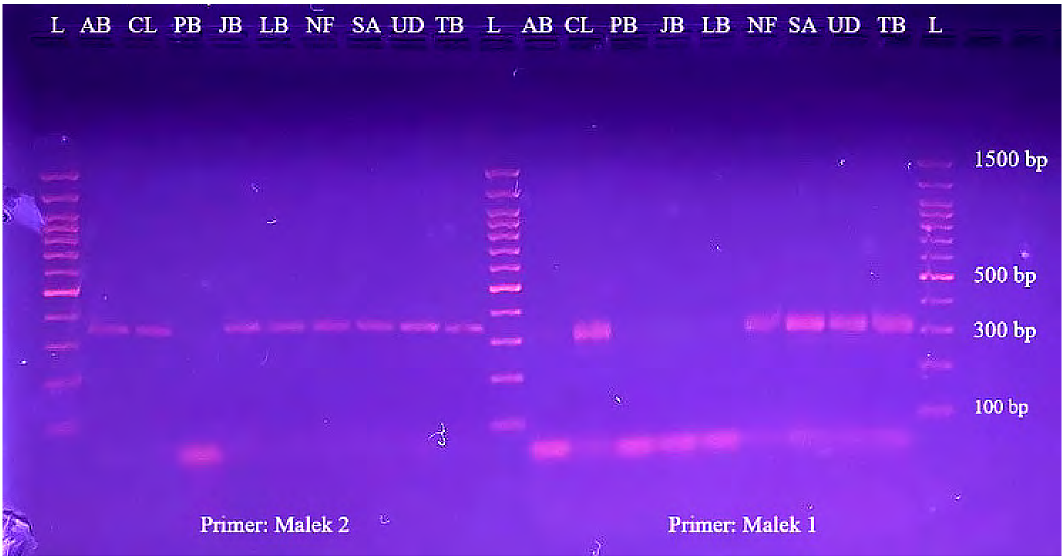

Fig. 2. Gel electrophoresis analysis of primers (Malek 1, Malek 2)

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Sequencing Results. The sequencing reactions indicated the exact identity after performing NCBI BLASTn for these PCR amplicons. Concerning the *DRF1* gene, the NCBI BLASTn engine showed up to 99% sequence similarity between the sequenced samples (assigned E1, E2, R1, R2, and R3) and the intended *DRF1* gene sequences of ***Triticum turgidum*** (GenBank acc. KM504244.1). As well, the NCBI BLASTn engine showed 100% sequence similarity between the sequenced samples (assigned S1, S2, S3, S4, S5) and the *NAC20L* gene sequences of ***Triticum aestivum*** (GenBank acc. XM_044571539.1). The genotype *T. turgidum durum* is one of the parents of bread wheat and durum wheat; it is a tetraploid wheat ($2n = 4x = 28$, *AABB*) [22]. The geographical distribution of wild wheat is in the Fertile Crescent region in southwest Asia, Palestine, Jordan, Lebanon, Syria, southern Turkey, northern Iraq, and southwest Iran [23, 24]. It can serve as one of the most important genetic resources to improve durum (*Triticum turgidum* L. ssp. *durum* (Desf.) and bread wheat (*Triticum aestivum* L.), and it has been used for allele mining to address various wheat breeding requirements, including, but not limited to, drought [25, 26] and salinity tolerance [27], as well as resistance to biotic stress factors. All the investigated genetic sequences were deposited in the NCBI web server, and unique accession numbers were obtained for all analysed sequences in Table 6.

Table 6

GenBank accession numbers for nucleotide sequences https://www.ncbi.nlm.nih.gov/genbank/

Locus	Accession. №	GenBank. №	Amplicon	Source	Primers	Strain	Samples	Genotypes
Seq1	PP873665	BankIt 2835921	DRF1	*T. turgidum*	Malek 1	Malek-E1	E1	AB
Seq2	PP873666					Malek-E2	E2	CL
Seq3	PP873667					Malek-R1	R1	NF
Seq4	PP873668					Malek-R2	R2	SA
Seq5	PP873669					Malek-R3	R3	TB
Seq6	PP873670		NAC20L	*T. aestivum*	Malek 2	Malek-S1	S1	CL
Seq7	PP873671					Malek-S2	S2	NF
Seq8	PP873672					Malek-S3	S3	SA
Seq9	PP873673					Malek-S4	S4	TB
Seq10	PP873674					Malek-S5	S5	UD

Source: compiled by M.H. Walli, F. Duksi, Z. Al-jubouri, M. Zargar, A. Alhasnawi.

Conclusion

Genotypes AB, NF, CL, UD, SA, and TB were tolerant to drought stress. The wild resource (*Triticum turgidum L.*) can be considered a source of drought tolerance genes and be relied upon in breeding and genetic engineering programs. The genes *DRF1* and *NAC20L* are responsible for wheat drought tolerance, and the primers Malek 1 and Malek 2 can be considered ideal for detecting these genes. DNA markers, such as ISSR-PCR markers, are a powerful tool for identifying the genetic diversity of wheat varieties. The 20 primers used were able to identify the genetic fingerprint of the twenty-three genotypes of wheat.

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