



Application of Inter-Simple Sequence Repeat Markers in Genetic Variance Detection of Proso Millet (*Panicum miliaceum* L.) Germplasm Cultivated in Kazakhstan

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Abstract

This study examined the genetic diversity and relationships among 67 proso millet genotypes, comprising different varieties from eight origin groups. *Panicum miliaceum* L. is a highly cultivated crop in Kazakhstan. However, there has been no comprehensive study in Kazakhstan to investigate the genetic diversity and phylogenetic relationships of millet using inter-simple sequence repeat (ISSR) markers. Genomic DNA from the proso millet genotypes was analyzed using ISSR markers. A total of 16 ISSR primers generated 4268 amplified DNA fragments, ranging in size from 43 to 4792 bp (base pairs). Of these fragments, 901 were monomorphic (29.7%), while 3367 were polymorphic (70.3%), with an average polymorphism of 78.8% per primer. Primers 834, 835 and 840 exhibited the highest polymorphism and were the most effective in distinguishing the genetic diversity of the proso millet accessions. Genetic diversity parameters were evaluated for the eight groups, revealing an average polymorphism information content (PIC) value of 0.124. Principal coordinate analysis (PCoA) and the phylogenetic tree demonstrated a wider genetic diversity among the 67 accessions from the eight groups. Analysis of molecular variance (AMOVA) revealed the presence of extensive genetic variability within groups and minor molecular difference among the studied groups. This research provides novel insights into germplasm classification, evolutionary relationships, genomics, and breeding of proso millet.

Keywords: Proso millet; Germplasm; Inter-simple sequence repeat markers; Genetic diversity.

Received: 18 March 2025; Revised: 10 April 2025; Accepted: 11 April 2025.

Article type: Research article.

1. Introduction

Proso millet (*Panicum miliaceum* L.), also known as common millet or broomcorn millet, is a primary annual crop that was domesticated approximately 10,000 years ago in China. Historically, it was widely cultivated in India, Nigeria, Niger and China.^[1] Although proso millet is considered a minor crop

compared to major cereals like wheat and barley, research in its genetics, genomics, and breeding is important.^[2] Notably, the haploid genome size of *P. miliaceum* is approximately 1.04 pg or 1017.2 Mb (1C value).^[3]

In other regions, such as Europe, North America, Asia, Australia, and Africa, proso millet is primarily used as fodder and birdseed, as well as a food source in certain parts of Asia.^[4-6] However, in Kazakhstan, millet holds a significant cultural value as a traditional ingredient, particularly in local dishes such as tary, talkan, and zhent. Archaeological evidence suggests that millet was first discovered in Kazakhstan and across Central Asia around 5,000 years ago, with some scientists believing it was the sole crop cultivated by locals for an extended period. In Western Kazakhstan, millet is the main primary cereal crop, valued for its drought and salt tolerance, as well as its flexibility in terms of sowing dates, making it an important crop in the dry steppe zone.^[7] Regardless of the impeccable environmental and health benefits, proso millet

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remains an under-researched and underutilized crop. Consequently, plant breeders across the globe are working to develop superior varieties using both conventional and advanced breeding techniques.^[8]

Panicum miliaceum, commonly known as proso millet, is a grain crop that exhibits valuable agronomic traits, including high drought and salinity tolerance, efficient water use, and a short life cycle.^[9] In studying the genetic diversity levels of proso millet, inter-simple sequence repeats (ISSRs) markers have proven beneficial. Research has demonstrated that ISSRs offer a reliable, rapid, and cost-efficient method for assessing genetic diversity among closely related cultivars, characterizing accessions, and identifying cultivars and varieties.^[10-12] The merits of ISSRs, including simplicity of use, reproducibility, and efficiency, make them a valuable tool for genetic diversity studies in proso millet.

ISSR markers represent a logical development of the simple sequence repeat (SSR) marker technique, where a single primer is designed from the SSR region.^[13,14] The application of ISSR markers is a quick, cost-effective, and highly reproducible technique based on polymerase chain reaction (PCR) amplification.^[15,16] This technology offers several advantages, including a high level of polymorphism, relatively low technology costs, and no requirement for sequence data for primer development. The ISSR is DNA fragments, typically ranging from 100 to 3000 bp in length, located between adjacent microsatellite regions with opposite orientations. The technique is also straightforward to perform.^[17] Notably, some studies have shown that ISSR markers can provide the highest level of discrimination between any pair of genotypes.^[18]

ISSR markers have been effectively utilized to evaluate the genetic diversity at both inter- and intra-specific levels in various major agricultural crops, such as rice,^[20,21] wheat,^[22-24] finger millet,^[25,26] safflower,^[27] sweet potato,^[28] and stevia.^[29] For instance, in a study focused on strawberry varietal identification, a single primer was sufficient to distinguish 30 strawberry varieties of diverse geographic and genetic origins.^[30] In another study, a Chinese germplasm collection of sea buckthorn (*Hippophae* L.) was analyzed using 15 ISSR primers, resulting in the identification of different cultivars with the same name and identical cultivars with different names.^[18] Furthermore, ISSR markers have been successfully used to distinguish between wild and domesticated species of salt-tolerant barley (*Hordeum spontaneum*).^[31]

A study analyzing the genetic diversity of 16 accessions with significant agronomic traits utilizing 3 sets of different molecular markers, comprising 54 ISSR, 40 sequence-related amplified polymorphism (SRAP) and 12 SSR markers.^[32] The results indicated that ISSR markers outperformed SRAP markers in terms of the average number of bands and the effective number of alleles.^[33] Thus, ISSR markers can be a valuable tool for analyzing different plant species, including proso millet, and can be applied in multiple ways.

Information on the genetic diversity within a given species'

germplasm collection, particularly for important traits like yield and quality, is essential for utilizing this diversity in crop improvement programs.^[34,35] In Kazakhstan, previous studies have investigated the genetic diversity of proso millet germplasm using SSR markers and single-nucleotide polymorphism (SNP) markers.^[36,37] Notably, ISSR markers have been employed to assess the genetic diversity of mutant proso millet plants obtained through colchicine treatment.^[38] However, there is a notable gap in the literature regarding the application of ISSR markers to study the genetic diversity of Kazakhstan proso millet germplasm from diverse origins. The purpose of the present study was: a) to evaluate the genetic diversity in proso millet collection consisting of 67 accessions from different ecological and geographical zones using ISSR markers; b) to identify ISSR markers that generate substantial polymorphism to improve millet breeding in the future. Hence, we established the level of genetic diversity across the proso germplasm at a fast turnaround time with the help of the fragment analyzer technique.

2. Materials and methods

2.1 Plant material

The germplasm of millet includes samples of Commonwealth of Independent States (CIS) countries, non-CIS countries, and local varieties. A total of 67 proso millet accessions adapted to Kazakhstan conditions with valuable traits were used in this study for polymorphism testing. These accessions were grouped into eight categories based on their geographical origins: 1-Kazakhstan (n=27), 2-India (n=6), 3-Russia (n=16), 4-Tajikistan (n=3), 5-Turkey (n=9), 6-Afghanistan (n=2), 7-Hungary (n=2) and 8-Ukraine (n=2) and the corresponding map was generated in Excel software (Fig. 1).

2.2 DNA extraction

The genomic DNAs were extracted from the young leaves of 2-week-old proso millet seedlings using a modified protocol based on Murray and Thompson.^[39] Specifically, 2×CTAB buffer was used for the extraction process. The concentration of the extracted DNA was calculated by measuring the absorbance of 1 µL of the sample at 260/280 nm using the NanoDrop 2000 spectrophotometer.

2.3 PCR analysis

A set of 16 ISSR primers, which had shown consistent results in the previous experiment, was selected for optimization of PCR conditions. The ISSR markers, representing dimer repeats, were procured from Zalma Ltd LLP. To achieve optimal amplification, different concentrations of template DNA were tested. The optimized PCR amplification reaction mixture had a total volume of 15 µL, consisting of 25 µL of 2×Master Mix for PCR (Vivantis, Malaysia), 23 µL of ddH₂O, 10 µM of 1 µL ISSR marker, and 1 µL of template DNA (approximately 30 ng/µL). Genomic DNA from all the proso millet genotypes was amplified using the 16 ISSR primers.



Fig. 1: Geographical distribution of 67 proso millet accessions.

The ISSR primers were selected based on the studies by Dvořáková *et al.*^[40] and used for the screening. PCR amplification was performed on the VeritiPro™ Thermal Cycler (Applied Biosystems, Singapore) under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at the optimal temperature for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 10 min.

2.4 Fragment analysis

Following amplification, the PCR products were loaded into

the 16-capillary system of the Fragment Analyzer™ Automated CE System. The results were then converted into a digital format using PROSize 3.0 software (Advanced Analytical Technologies, Ankeny, IA, USA). Fragment analysis was laid out to determine allele sizes within the range of 35 bp to 5000 bp using the dsDNA 915 Reagent Kit (Agilent Technologies, Inc., Iowa, USA). A total of 16 ISSR markers were used to estimate the genetic diversity among the tested proso millet accessions, with each assay replicated 3 times. Detailed information about the ISSR primers used in this study is presented in Table 1.

Table 1: The information of 16 ISSR primers and the polymorphic information content (PIC).

Marker names	Sequence 5'→3'	Optimized annealing temperature (°C)	PIC
ISSR 807	(AG) ₈ T	42.0	0.126
ISSR 808	(AG) ₈ C	47.4	0.154
ISSR 809	(AG) ₈ G	46.3	0.125
ISSR 810	(GA) ₈ T	42.0	0.108
ISSR 811	(GA) ₈ C	43.2	0.117
ISSR 816	(CA) ₈ T	43.2	0.124
ISSR 817	(CA) ₈ A	53.0	0.105
ISSR 819	(GT) ₈ A	47.4	0.066
ISSR 820	(GT) ₈ C	50.1	0.031
ISSR 822	(TC) ₈ A	45.8	0.083
ISSR 823	(TC) ₈ C	47.4	0.091
ISSR 826	(AG) ₈ C	53.0	0.147
ISSR 834	(AG) ₈ YT	45.8	0.193
ISSR 835	(AG) ₈ YC	45.7	0.185
ISSR 840	(GA) ₈ YT	45.7	0.208
ISSR 841	(GA) ₈ YC	45.8	0.131

2.5 Molecular statistical analysis

To analyze the genetic diversity of the proso millet populations, the following parameters were calculated using GenAlEx6.5 for MS-Excel software:^[41,42] observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), unbiased expected heterozygosity (uHe) and percentage of polymorphic loci (P). The polymorphism information content (PIC) of the ISSR markers was calculated to differentiate proso millet populations, using Eq. (1):

$$PIC = 1 - \sum p_i^2 \quad (1)$$

where p_i is the frequency of the i th band in the set of proso millet accessions.^[43] Phylogenetic relationships between the studied millet samples were inferred by clustering and constructing a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA) method implemented in the molecular evolutionary genetics analysis (MEGA)

software package.^[44]

3. Results and discussion

3.1 Genetic diversity of germplasm between groups

This study employed molecular genetic analysis using 16 ISSR markers to examine the genetic diversity of a proso millet collection. These markers are versatile tools applicable in various areas of genetics, genomics, and plant research. The genotypes of all accessions were categorized into eight groups based on their geographical origins. The PIC values ranged from 0.031 (ISSR 820) to 0.208 (ISSR 840), with an average PIC value of 0.124. A total of 4268 fragments were amplified by the 16 ISSR primers from eight populations, with an average of 266.75 bands per primer. The size of the amplified products ranged in size from 43 to 4792 bp. Statistical analysis showed that 3367 bands were polymorphic, while 901 bands were monomorphic. The molecular size of the allele and the average number of fragments in different groups have been

Table 2: Analysis of allelic variation range and mean number of bands.

Markers	AVR and ANF	Groups							
		1	2	3	4	5	6	7	8
ISSR 807	AVR	47-4738	163-3223	45-4289	177-853	145-1543	209-4112	174-1001	50-740
	ANF	21	27	22	19	23	27	26	17
ISSR 808	AVR	43-4733	210-3981	151-4080	188-4440	43-4340	208-853	187-1493	204-997
	ANF	28	27	28	26	24	31	35	26
ISSR 809	AVR	48-4596	138-4495	146-1326	214-782	42-3869	220-666	145-4394	148-4334
	ANF	19	25	20	22	22	10	27	21
ISSR 810	AVR	46-4756	211-4445	47-4489	210-935	207-4067	211-1286	214-1386	210-941
	ANF	21	21	17	23	25	12	11	33
ISSR 811	AVR	43-4506	292-4549	281-4742	376-3216	291-1958	487-1397	281-3560	378-1554
	ANF	15	19	14	19	16	14	15	15
ISSR 816	AVR	43-4535	42-4093	292-3210	357-2473	292-4605	294-4489	430-3954	293-2681
	ANF	18	21	17	18	17	17	18	17
ISSR 817	AVR	42-4043	41-3235	42-4638	42-3256	40-1630	281-1785	42-3660	50-1439
	ANF	8	8	8	7	8	9	17	11
ISSR 819	AVR	42-4683	42-4386	42-3971	42-1380	40-4743	228-1759	42-2782	52-4367
	ANF	9	9	8	5	9	17	10	12
ISSR 820	AVR	44-4736	202-4319	41-4758	44-2546	41-2131	181-1875	46-4099	205-1795
	ANF	18	20	17	19	19	17	16	22
ISSR 822	AVR	44-4707	41-4345	43-4742	156-3311	145-4087	143-4242	152-2552	139-4466
	ANF	12	13	12	14	12	17	14	10
ISSR 823	AVR	199-4786	244-4090	41-3983	41-4072	47-6983	249-4286	249-4554	41-4482
	ANF	16	14	16	9	17	7	20	12
ISSR 826	AVR	50-4612	129-4418	129-4748	183-2614	130-4631	236-4554	131-4592	182-4670
	ANF	27	28	27	29	28	27	29	23
ISSR 834	AVR	124-4717	145-4738	124-4556	193-4354	125-4556	145-4778	145-4697	191-3930
	ANF	38	40	37	38	38	35	26	35
ISSR 835	AVR	45-4485	108-4608	41-4588	129-4031	108-4608	132-4773	108-4794	82-1077
	ANF	34	34	36	33	36	33	28	37
ISSR 840	AVR	48-4792	144-4646	138-4521	145-4146	43-3688	146-4396	144-4084	138-1465
	ANF	40	43	41	30	41	41	43	37
ISSR 841	AVR	153-4639	160-4109	47-4494	160-674	59-3964	163-3964	161-666	160-3868
	ANF	21	20	21	21	21	22	22	23

Note: AVR - Allelic variation range (bp); ANF - Average number of fragments

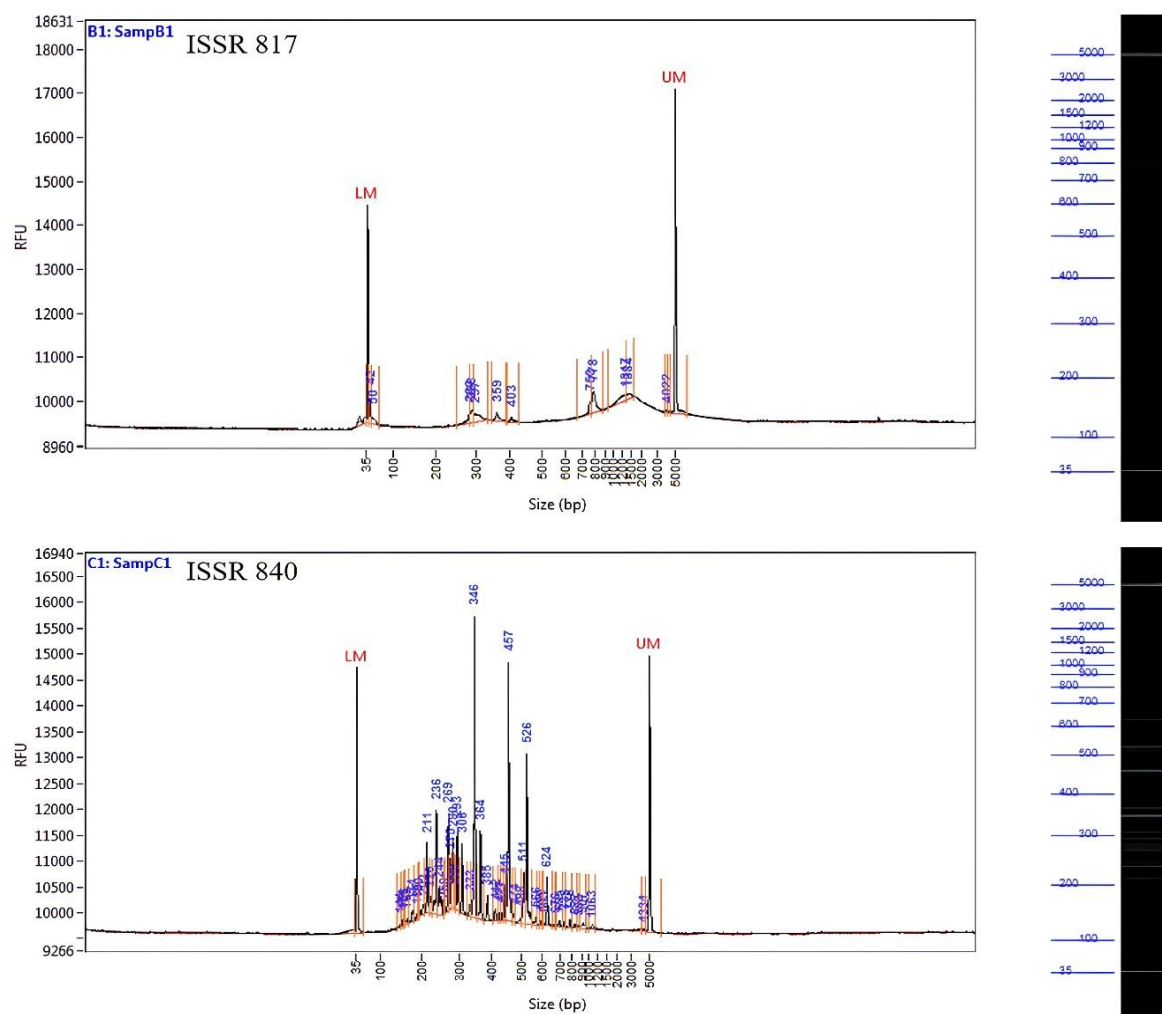


Fig. 2: Allelic variation in proso millet germplasm using ISSR markers 817 and 840 analysed with capillary electrophoresis (B1, C1-samples of genotypes).

ranged depending on ISSR markers. The maximum level of average number of fragments was produced by ISSR 840 among all 16 markers. Allelic variation range (AVR) and average number of fragments (ANF) are shown in Table 2.

The mean number of scorable markers produced per primer ranged from 7 to 43, with an average of 266.75 bands per primer. The markers ISSR 840 and ISSR 834 generated the highest number of amplified bands, with 316 and 287, respectively. In contrast, the markers ISSR 817 and ISSR 819 showed the lowest number of amplified DNA segments, with 76 and 79, respectively. Representative capillary electrophoresis patterns obtained using primers 817 and 840 are illustrated in Fig. 2.

The mean polymorphism value for ISSR loci was 78.9%. Primer ISSR 840 exhibited the highest polymorphism value (89%), whereas primer ISSR 823 showed the lowest value (57.6%). A high percentage of polymorphic bands was observed in groups 6 and 8, with 91.0 and 90.2% polymorphism, respectively. In contrast, groups 1 and 3 displayed a lower percentage of polymorphic bands, with 63.9 and 67.9% polymorphism, respectively. The percentage of analyzed polymorphic bands for each group is presented in

Table 3.

The genetic variability parameters, like the number of observed (N_a) and effective number of (N_e), Shannon's information index (I), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), and percentage of polymorphic loci (P), were computed and summarized in Table 4.

The mean observed and effective numbers of alleles (N_a and N_e) across all groups were 0.723 and 1.092, respectively. The highest number of effective alleles was obtained from the Hungary group (1.107), while the Turkey group recorded the lowest value (1.085). The mean Shannon index, a measure of genetic diversity, was 0.111 for all origin groups. The Russia group had the highest Shannon index value (0.150), and the Afghanistan group had the lowest value (0.084). The expected heterozygosity (H_e) was highest in the Russia group (0.076) and lowest in the Ukraine group (0.057). The unbiased expected heterozygosity (uH_e) ranged from 0.07 to 0.083, with an average of 0.076. The average percentage of polymorphic loci (P) for the total population was 36.01. The Kazakhstan population showed the highest percentage of polymorphism (78.87%) and the highest number of observed

Table 3: The percentage of polymorphic bands generated by 16 ISSR primers from 8 origin groups of proso millet

Markers	Groups								Mean (%)
	1	2	3	4	5	6	7	8	
ISSR 807	60.5	81.8	66.6	70.3	63.8	100.0	100.0	58.6	75.2
ISSR 808	67.0	84.3	73.6	78.7	66.6	100.0	87.5	96.2	81.7
ISSR 809	48.7	75.7	68.9	88.0	68.7	100.0	81.8	100.0	78.9
ISSR 810	52.2	65.6	47.2	71.8	78.1	75.3	64.7	100.0	69.3
ISSR 811	68.1	79.1	77.7	95.0	80.0	100.0	100.0	100.0	87.4
ISSR 816	64.2	80.7	68.0	90.0	65.3	100.0	90.0	100.0	82.2
ISSR 817	50.0	72.7	53.3	63.6	72.7	100.0	94.4	100.0	75.8
ISSR 819	50.0	64.2	53.3	62.5	75.0	100.0	100.0	100.0	75.6
ISSR 820	69.2	74.0	53.1	79.1	73.0	100.0	84.2	95.6	78.5
ISSR 822	66.6	86.6	63.1	82.3	75.0	89.4	77.7	90.9	78.9
ISSR 823	55.1	60.8	61.5	52.9	51.5	53.8	71.4	54.5	57.6
ISSR 826	79.4	96.5	84.3	90.6	82.3	84.3	96.6	95.8	88.7
ISSR 834	84.4	86.9	86.0	97.4	88.3	87.5	60.4	92.1	85.3
ISSR 835	62.9	82.9	73.4	82.5	85.7	89.1	90.3	86.0	81.6
ISSR 840	83.3	93.4	87.2	69.7	91.1	93.1	100.0	94.8	89.0
ISSR 841	61.7	80.0	70.0	77.7	75.0	84.6	91.6	79.3	77.4
Mean	63.9	79.0	67.9	78.2	74.5	91.0	86.9	90.2	78.9

Table 4: Genetic diversity parameters of proso millet origin groups

Group	Na	Ne	I	He	uHe	P, %
1	1.570±0.012	1.080±0.001	0.140±0.002	0.072±0.001	0.073±0.001	78.87
2	0.680±0.015	1.090±0.002	0.120±0.003	0.069±0.002	0.075±0.002	34.44
3	1.389±0.014	1.089±0.001	0.150±0.002	0.076±0.001	0.078±0.001	69.47
4	0.363±0.012	1.089±0.003	0.091±0.003	0.058±0.002	0.070±0.002	18.02
5	0.889±0.015	1.085±0.002	0.125±0.002	0.068±0.001	0.072±0.001	44.47
6	0.285±0.011	1.098±0.004	0.084±0.003	0.058±0.002	0.077±0.003	13.89
7	0.311±0.011	1.107±0.004	0.091±0.003	0.063±0.002	0.083±0.003	15.11
8	0.283±0.011	1.098±0.004	0.084±0.003	0.057±0.002	0.076±0.003	13.82
M±SD	0.723±0.005	1.092±0.001	0.111±0.001	0.065±0.001	0.076±0.001	36.01

Note: Na – Observed number of alleles; Ne – Effective number of alleles; I – Shannon's Information index; He – Expected heterozygosity; uHe – Unbiased Expected Heterozygosity; P – percentage of polymorphic loci. M-mean; SD-Standard deviation

alleles (1.570). The Russia (69.47%), Turkey (44.47%), and India (34.44%) groups showed higher percentages of polymorphism. However, the groups from Tajikistan (18.02%), Hungary (15.11%), Ukraine (13.82%), and Afghanistan (13.89%) displayed the least polymorphisms.

The assessment of germplasm diversity is imperative to facilitate genetic improvement of crops. Despite the significance of proso millet in the Republic of Kazakhstan, there is still relatively limited information on its genetic diversity and phylogenetic relationships based on molecular markers.^[45,46] ISSR markers have been widely used for germplasm characterization due to their better reproducibility, ability to detect a large number of polymorphic and discriminant fragments, and relatively low costs. ISSR markers are particularly useful for assessing genetic diversity among species or varieties within the same genus and are also considered useful for gene mapping studies.^[47-49] This study employed ISSR to examine the genetic diversity and relationships among 67 proso millet varieties originating from

different ecological and geographical zones. An initial screening using the selected 16 ISSR markers was conducted to verify the selected marker set's capacity to reveal sufficient polymorphism in the studied genotypes. The analysed primers produced well-amplified bands, ranging from 43 to 4792 bp.

According to the classification of Botstein *et al.*^[50] (1980), the PIC value obtained in our study, four markers (ISSR 819, ISSR 820, ISSR 822, and ISSR 823) were low informative, and seven ISSR primers (ISSR 807, ISSR 809, ISSR 810, ISSR 811, ISSR 816, ISSR 817, and ISSR 841) were moderately informative. Five markers (ISSR 808, ISSR 826, ISSR 834, ISSR 835, and ISSR 840) were highly informative and therefore very useful for studies of genetic diversity in proso millet.^[50]

The level of polymorphism based on ISSR markers is highly variable according to the studied species. Polymorphism level of 95.93% was found for *Arabidopsis thaliana*,^[51] 42.53% for *Omphalogramma souliei* Franch,^[52] 56.05% for *Emmenopterys henryi*,^[53] and 81.82% for *Vernicia*

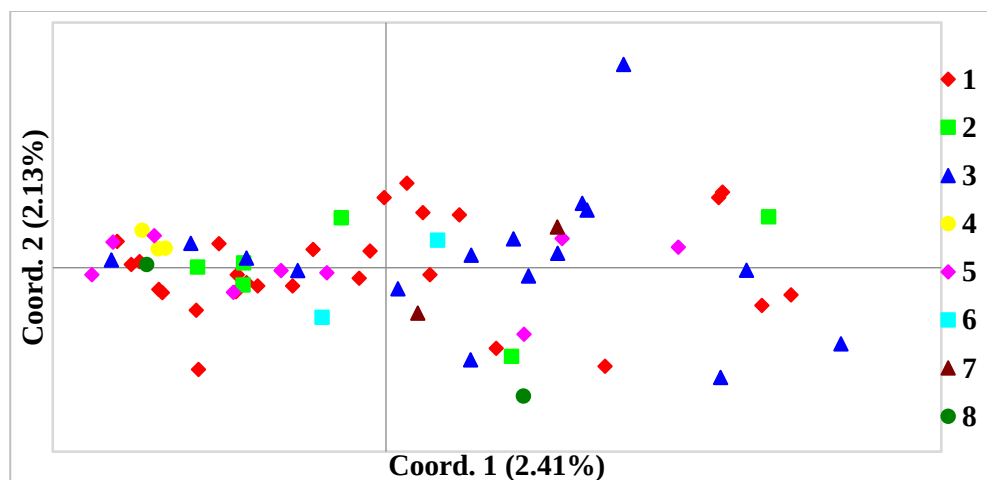


Fig. 3: Principal component analysis of the proso millet germplasm based on ISSR markers. Groups: 1-Kazakhstan, 2-India, 3-Russia, 4-Tajikistan, 5-Turkey, 6-Afghanistan, 7-Hungary, and 8-Ukraine.

fordii.^[54] The study results, which showed a mean percentage of polymorphic bands of 78.8% for proso millet, agreed with multiple studies on the molecular characterization of germplasm using ISSR markers. Notably, the ISSR 840 marker exhibited particularly high polymorphism, with a value of 89%. These results are in accordance with Elias M.S. *et al.*, according to their research, ISSR markers showed high polymorphism and generated a total of 13 alleles.^[55]

3.2 Genetic relationship between proso millet accessions

The relationships among the 8 groups were assessed using principal component analysis (PCoA). The analysis revealed two genetically distinct clusters based on genetic diversity among the groups. The first two principal coordinates (P1 and P2) explained 2.41% and 2.13% of the total variation, respectively. The results of the PCoA are illustrated in Fig. 3.

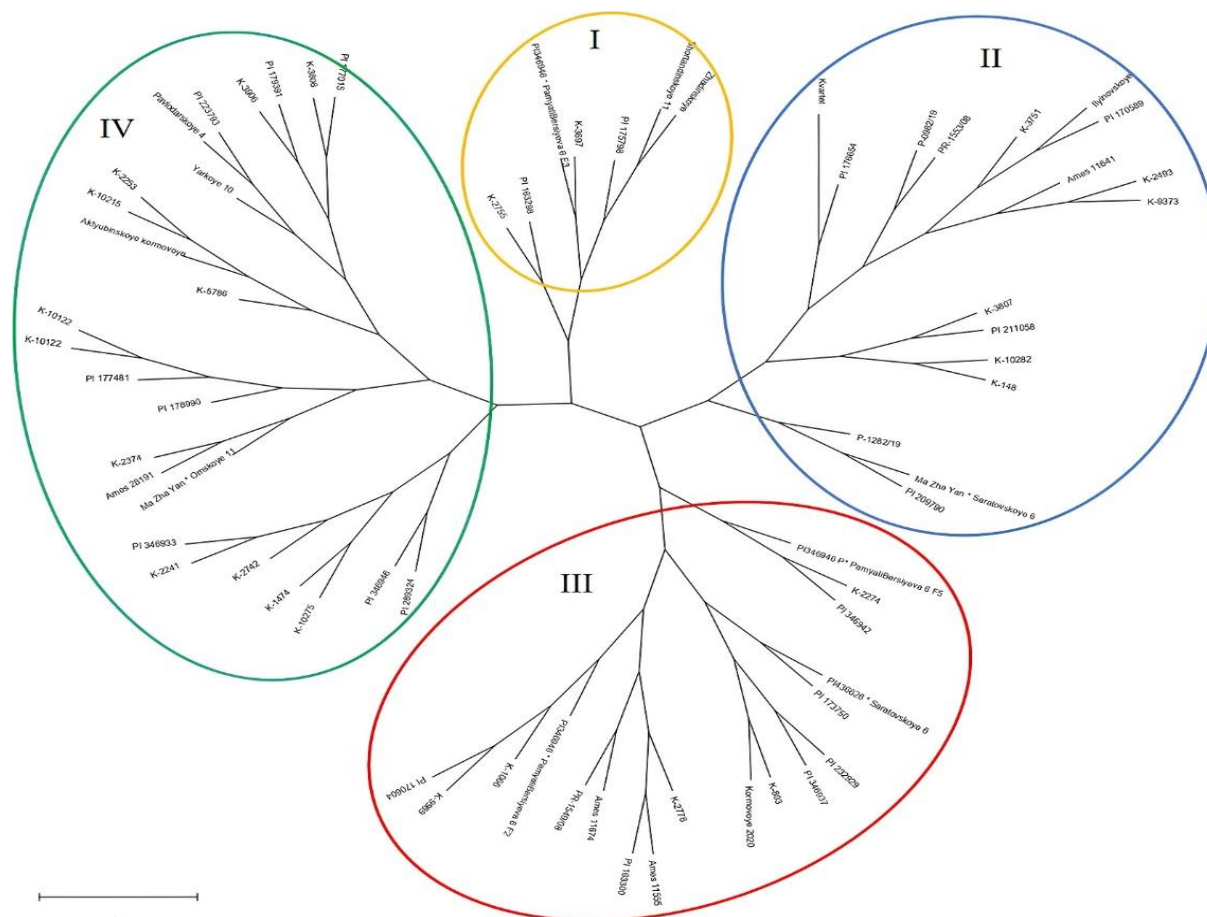


Fig. 4: Phylogenetic tree of 67 proso millet genotypes based on the data of the ISSR markers.

Table 5: AMOVA test of the studied groups

Source	df	SS	MS	EV	%
Among groups	7	4289.205	612.744	22.753	7%
Within groups	126	36054.884	286.150	286.150	93%
Total	133	40344.090	-	308.903	100%
Stat	Value	P (rand >= data)			
Fst	0.074	0.001			

Note: df – degree of freedom; SS – sum of squared observations; MS – mean of squared observations; EV – estimated variance.

A score plot of the first two principal coordinates (Coord. 1 and Coord. 2) showed the relationships among the 67 proso millet accessions. The PCoA incorporated origin data and revealed that most Kazakhstan-origin genotypes were genetically close to the India, Turkey, and Russia groups, with accessions from these groups distributed across the whole spectrum. The Afghanistan and Hungary groups occupied an intermediate position, closer to the Kazakhstan and Russia groups. Conversely, the Ukraine accessions were positioned at the bottom right of the plot, while the Tajikistan group accessions were located at the top left, near the Kazakhstan, Turkey, and Russian groups. To further clarify the phylogenetic relationships among all 67 accessions from eight groups, a phylogenetic tree was constructed using MEGA software (Fig. 4).

The phylogenetic tree clustered the 67 accessions into four groups with mixed accessions. Cluster I (yellow) consisted of 7 millet accessions from groups 1-5. Cluster II (blue) comprised 17 genotypes, with 8 belonging to the group 1, and represented groups 1-6, similar to Cluster I. Cluster III (red) primarily contained group 1 accessions (8 genotypes), followed by group 2 and 3 (3 genotypes each), then the group 5 (2 genotypes), and single genotype representatives from groups 7 and 8. Cluster IV (green) had 25 accessions, including 8 from group 1, 3-4 from group 5, and single genotype representatives from groups 6, 7, and 8. Clusters III and IV exhibited wider genetic diversity than Clusters I and II, possibly influenced by the group size. Although molecular phylogenetic analysis divided all genotypes into 4 clusters, these groups were not classified according to their origin areas.

Since an ISSR may be a conserved or non-conserved region, this technique is not useful for distinguishing individuals, but rather for phylogeographical analyses or maybe delimiting species.^[56] To investigate the genetic relationships among the 67 proso millet accessions, we employed two multivariate molecular analysis methods: PCoA and phylogenetic tree construction. Each method was designed to organize the data on a different basis and to be used comparatively to elucidate relationships. The polymorphisms identified by these markers are a valuable parameter for understanding genetic differences between groups of different origins. ISSR results based on PCoA revealed genetic relatedness among the 67 accessions. In general, genetically close individuals are often clustered together.^[57]

The study analysis using both PCoA and phylogenetic tree

construction showed that the ISSR technique did not discriminate proso millet genotypes according to the origin. These results agree with those of Ajie (2025) and Ceballos-Aguirre *et al.* (2017),^[58,59] where the ISSR markers used did not group the tomato introductions according to the geographical origin from which they were collected. This may be attributed to germplasm exchange within the region and the fact that all accessions belong to the same species, *Panicum miliaceum* L. Analysis of molecular variance (AMOVA) revealed that the geographical position of the breeding site had a minimal impact on the diversity of proso millet genotypes, accounting for only 7% of the total variation. In contrast, the majority of the variation (93% of the total variation) was found among the genotypes themselves. The fixation index (Fst) value of 0.074 ($p < 0.001$) indicated a significant level of genetic differentiation among the groups (Table 5).

Minor difference value (7%) obtained by AMOVA test likely stems from the genetic differences between the groups. The results indicate that the geographical origin was not influenced by the classification of germplasm. The study showcases the effectiveness of the ISSR marker system in uncovering informative polymorphisms within proso millet germplasm.

4. Conclusion

Proso millet is the main ancient cereal crop in Kazakhstan. The use of ISSR markers in evaluating and identifying proso germplasm is vital in genetic and breeding studies. In this study, the fragment analysis method was used to assess the genetic diversity levels of Kazakhstan germplasm for the first time. From the results obtained in this study, ISSR 834, ISSR 835, and ISSR 840 markers have a high efficiency in differentiating proso millet accessions of *Panicum miliaceum* L. cultivated in Kazakhstan. The profiles generated by primer ISSR 840 contained the largest number of polymorphic bands (316 bands). The high genetic variability indicates the valuable genetic potential present in proso germplasm, which could be used for future breeding programs. The phylogenetic tree divided the 67 proso millet accessions into 4 main clusters, where each cluster was represented by different origin genotypes. This study demonstrates that ISSRs provide a rapid and reproducible method for assessing genetic diversity among proso millet groups of various origins. The findings indicate that the ISSR marker system is efficient in discriminating genotypes at the molecular level.

Acknowledgments

This work was supported by the College of Agronomy of the Northwest A&F University. The authors express their gratitude to Hu Yin-Gang, Professor of Crop Genetics and Breeding for providing opportunity to conduct the fragment analysis in his department and for reviewing this manuscript. This work was carried out within the framework of the scientific project AP22785049, “Improvement of the breeding process based on chemical mutagenesis to obtain early-ripening mutant forms of millet (*Panicum miliaceum* L.)” (2024-2026). Grant funding for the research work was provided by the “Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan” State Institution.

Conflict of Interest

There is no conflict of interest.

Supporting Information

Not applicable.

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