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PROSO MILLET (*PANICUM MILIACEUM* L.) GERMPLASM ASSESSMENT FOR YIELD-RELATED TRAITS USING SSR MARKERS

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SUMMARY

Proso millet (*Panicum miliaceum* L.) has a diverse germplasm, comprising 152 accessions that differ in productivity. These received evaluation during 2024 and 2025 for yield indicators and genetic diversity under field conditions in the North Kazakhstan Region, Kazakhstan. The accessions underwent grouping based on their productivity, with the efficiency of 16 SSR markers evaluated for genotyping the proso millet genotypes. According to analysis, 9% of the total genetic variability was evident among the yielding genotype groups, while 91% variability was within groups. The fixation index showed a moderate, however, significant genetic ranking between low-, medium-, and high-yielding genotypes ($F_{st} = 0.091$). AMOVA showed that 9% of the total genetic variation was in association with differences among the yield groups, while observing 91% within groups. Correlation analysis of the SSR markers and agronomic traits sought to identify the promising markers found capable of determining productive tillering in various proso millet genotypes. Three SSR markers (SSR 409, SSR 365, and SSR 331) had moderate to significant correlation with the studied traits; the marker SSR 409 also had the maximum PIC value (0.595). The PCoA has proven capable of separating low-, high-, and medium-yielding genotypes and possibly helped in reducing the duration of the selection process.

Keywords: Proso millet (*P. miliaceum* L.), germplasm, genetic diversity, agronomic traits, grain yield, SSR markers

Key findings: The SSR markers established significant associations with viable agronomic traits and served as useful tools for targeted breeding aimed at improving productivity and developing genetically enhanced proso millet (*P. miliaceum* L.) genotypes.

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INTRODUCTION

Proso millet (*Panicum miliaceum* L.), also known as common millet, is one of the oldest cultivated cereals. However, nowadays, proso millet is widespread worldwide, grown across Asia, Europe, North America, Australia, and Africa (Vetriventhan *et al.*, 2019). Proso millet is a short-season annual grass, belonging to the family Poaceae. Though it seemed a minor cereal compared with rice, wheat, and maize, millets rank as the sixth most important cereal crop globally in terms of cultivation area and production (Habiyaemye *et al.*, 2017). Proso millet in particular is precious for its fast maturity (often 60–90 days) and adaptability to various environmental and soil conditions (Baltensperger, 2002). In many countries, proso millet has become an essential food and fodder grain (Das *et al.*, 2019). Its rich nutritional profile and gluten-free characteristics have increased its value in functional food systems (Saleh *et al.*, 2013).

Millet species, including *P. miliaceum*, owned the most crucial feature of drought tolerance and ability to grow under arid and semi-arid environments. Proso millet has a low water requirement and can produce grains more efficiently per unit of moisture than various other crops (Rysbekova *et al.*, 2025). Its short growth cycle and C₄ photosynthetic pathway enable it to endure drought conditions that would damage other crops (Shi *et al.*, 2022). These viable traits prove proso millet reliable for regions with minimal rainfall, poor soils, and high-temperature conditions. The Kazakhstan climate is characteristically semi-arid (Lioubimtseva and Henebry, 2009), which also has a long history of proso millet cultivation. Proso millet is particularly well adapted to the dry-steppe conditions of Central Asia, having been cultivated in the region for a longer period.

In plant genetics and breeding research, simple sequence repeat (SSR) markers have reached distinction as an indispensable tool for DNA analysis. The SSR markers are short, tandemly repeated nucleotide sequences scattered throughout the genome (Ellegren, 2004). The SSR markers

own several beneficial properties: SSRs are co-dominant markers, multi-allelic, abundant, and evenly distributed in most plant genomes and can undergo assays with relatively low cost and labor. In comparison to other markers—restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP)—the SSR markers revealed greater levels of polymorphism and appeared more reproducible, proving them powerful for assessing genetic diversity in crop plants.

Plant breeders' primary task is to introduce the highly adaptive and high-yielding proso millet cultivars and ensure their genetic potential. However, the application of high-throughput indirect phenotypic data in combination with SSR markers to investigate the genetic diversity of proso millet requires their extensive use, including the most effective markers in this group. Addressing this gap led to the use of 16 SSR markers to evaluate the genetic diversity of a proso millet germplasm comprising 152 genotypes differing in productivity and grown under the dry steppe zone of Kazakhstan. The following research aimed to study the genetic diversity using 16 SSR markers in proso millet germplasm differing in productivity and evaluate the yield components. These include 1000-seed weight, panicle seed weight, productive tillering, and the grain yield harvested under North Kazakhstan conditions during 2024 and 2025. The study also sought to evaluate the relationship between SSR markers and agronomic traits of proso millet genotypes with varying productivity levels. These results will enable the possibility of integrating indirect phenotypic data based on agronomic traits and genotypic data based on SSR markers into the assessment of the genetic diversity of proso millet genotypes for yield-related traits.

MATERIALS AND METHODS

Genetic materials

The proso millet (*P. miliaceum* L.) germplasm

comprised 152 cultivars, procured from different ecological origins, including Central and Eastern Europe and Central, Southern, and Eastern Asia. The evaluated genotypes also belonged to different collections such as the United States Department of Agriculture (USDA) (56 genotypes), the Kazakhstani collection (33), and N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) (63).

Field experiment

The field study proceeded at the A.I. Barayev Research and Production Centre for Grain Farming, Akmola Region, Kazakhstan. The 152 proso millet varieties entailed sowing in a selection crop rotation after fallow. Proso millet seed collection, as sown, used the SSFK-7 seed drill at a seeding rate of 300 seeds/m² (3 million viable seeds), in duplicate, with a plot area of 2 m² and a systematic layout. The study used the region's traditional agrotechnology in the field experiment. Crop growing was under rainfed conditions without irrigation. The completion of harvest occurred when all accessions in the collection had reached full maturity. The data recorded were on various plant structure and productivity traits. These included general yield (GY), productive tillering (PT), the number of seeds per panicle (NSPP), seed weight per panicle (SWPP), the number of seeds per plant (NSPA) and seed weight per plant (SWPA), 1000-seed weight (TSW), and plant stand survival to harvest (PSSH).

SSR marker data analysis

Genomic DNA of each proso millet genotype gained isolation using the CTAB (cetyltrimethylammonium bromide) method. In molecular and genetic studies and assessing polymorphism, the SSR markers' use took place at Saken Seifullin Kazakh Agrotechnical University. A total of 16 SSR markers selected for the said research had an initial description by Wang *et al.* (2021) (Table S1).

Identification of PCR amplification products

The amplification of polymerase chain reaction (PCR) continued in the Applied Biosystems SimpliAmp thermocycler. Each PCR tube contained 6.25 µl of Taq 2X Master Mix, 4.25 µl of ddH₂O, 0.6 µl of reverse primer, 0.6 µl of forward primer, and 20 ng of DNA. The conditions of the temperature cycle followed the protocol by Wang *et al.* (2021): denaturation at 95 °C for 5 min, followed by 35 cycles at 40 s at 95 °C, 30 s at the annealing temperature, 30 s at 72 °C, and finally an extension at 72 °C for 10 min. Detailed information on the markers such as sequences and annealing temperatures is available in Table S1. Using the 1% agarose gel electrophoresis with a non-toxic dye sought to visualize the products of PCR amplification, with the migrated fragments visualized in Gel Doc XR System.

Statistical analysis

The research employed Microsoft Excel to study phenotypic data, while engaging RStudio 4.4.1 with the adegenet, pegas, and corplot packages sought to analyze and visualize phenotypic data. Electrophoresis results received scoring with GelAnalyzer 6.5, with the fragments' sizes determined by comparison with a molecular weight marker. Then, creating a binary data matrix enabled treating each distinct fragment size as an allele. GenAlEx 6.5 software served to calculate allele frequencies and estimate the genetic diversity parameters. The calculation of the polymorphism information content (PIC) of the SSR markers to differentiate proso millet populations used the following formula:

$$PIC = 1 - \sum_{i=1}^N P_i^2$$

Where P_i is the frequency of the i -th band in the set of proso millet accessions. Correlation analysis between the SSR markers and the quantitative phenotypic traits, when performed, used Pearson's correlation coefficient.

Table 1. Agronomic traits of the proso millet germplasm by productivity groups.

Groups	Descriptive stat	Seed weight per panicle (g)	1000-seed weight (g)	Productive tillering, pcs	Plant stand to survival, pcs	General yield (g)
1 (low)	Min/Max	0.79/17.44	4.52/12.42	1.4/2.76	70/98	95.95/177.71
	Range	16.55	7.90	1.36	25	81.76
	Mean	6.47	8.63	3.02	87.55	128.20
	SD	2.71	1.31	1.31	7.21	18.35
	CV	0.42	0.15	0.43	0.08	0.14
2 (medium)	Min/Max	2.71/12.36	5.645/12.98	1.4/2.65	70/96	180.1/350.33
	Range	9.65	7.33	1.25	23.0	170.23
	Mean	6.13	9.11	2.71	81.97	261.71
	SD	2.30	1.63	1.22	1.58	54.30
	CV	0.38	0.18	0.45	0.01	0.210
3 (high)	Min/Max	2.71/16.99	5.87/12.41	1.3/3.11	80/93	360.25/759.79
	Range	14.28	6.54	1.81	14	399.53
	Mean	6.46	9.05	2.61	84.15	476.29
	SD	3.32	1.54	0.98	0.54	101.17
	CV	0.515	0.17	0.25	0.007	0.21

Note: SD - Standard deviation, CV - Coefficient of variation

RESULTS

Agronomic traits based on productivity

According to yield data, the classification of proso millet (*P. miliaceum* L.) accessions totaled 152. Based on the harvested yield (g/m²), dividing the variety samples reached three groups: low (< 200 g/m²), medium (200–400 g/m²), and high-yielding (> 400 g/m²). Thus, 83 low-yielding variety samples were evident in Group 1, 49 medium-yielding ones in Group 2, and 20 variety samples emerged as high-yielding and belonged to Group 3 (Table 1). The proso millet variety samples underwent comparison based on the quantitative agronomic traits, such as productive tillers per plant, seed weight per panicle (g), 1000-seed weight (g), plant stand survival to harvest (%), and grain yield (g/m²).

In Group 3 genotypes, there was more stability, and many genotypes showed higher values for yield-related traits. Although Group 1 showed a good average result, it contained numerous low-yielding cultivars. The calculation of coefficient of variation (CV) also assessed variability among the proso millet genotype productivity groups. According to the

coefficient of variation obtained for productivity groups of genotypes, the seed weight per panicle (CV = 0.515) and grain yield (CV = 0.21) were the most promising variables in Group 3, and 1000-seed weight (CV = 0.18), productive tillering (CV = 0.45), and plant stand survival to harvest (CV = 0.01) for Group 2.

Genotyping using SSR Markers

The polymorphism analysis of 152 proso millet genotypes proceeded using 14 pairs of SSR primers (Table 2), since two markers, SSR 195 and SSR 232, did not produce any amplification product, and therefore, they were excluded from further analysis. The majority of the loci exhibited moderate levels of polymorphism. The marker SSR 409 emerged as highly informative. Following the obtained results, all the groups had the same percentage of polymorphic loci (78.6%). According to other indicators, Group 1 was distinguishable with higher variability than the other two groups, whereas Group 2 exhibited the lowest variability. Gene frequencies helped to calculate the PIC. In the analyzed markers, five primers appeared as monomorphic, viz., SSR 460, SSR 404, SSR 386, SSR 232, and

Table 2. Detailed information on SSR markers in proso millet.

Locus	Observed allele size in proso millet (bp)	Number of alleles	Number of polymorphic bands	Polymorphism (%)	PIC
SSR 394	145, 230	2	2	100	0.411
SSR 430	130, 150, 185	3	3	100	0.374
SSR 458	175, 220, 270, 725	4	4	100	0.477
SSR 420	215, 240, 300	3	3	100	0.359
SSR 365	150, 190	2	2	100	0.304
SSR 448	155, 190	2	2	100	0.388
SSR 357	140, 280	2	2	100	0.480
SSR 384	205, 325	2	2	100	0.261
SSR 460	210	1	0	0	0
SSR 409	160, 200, 240, 625	4	4	100	0.595
SSR 404	250	1	0	0	0
SSR 203	175, 195, 1100	3	3	100	0.467
SSR 331	225, 300	2	2	100	0.293
SSR 386	250	1	0	0	0
SSR 195	not detected	-	-	-	-
SSR 232	not detected	-	-	-	-

Table 3. Allelic patterns for codominant data in proso millet.

Group	Na	Ne	I	He	uHe	P (%)
1	2.636±0.244	1.779±0.119	0.661±0.070	0.410±0.043	0.413±0.043	78.6
2	2.000±0.191	1.699±0.170	0.531±0.105	0.346±0.068	0.350±0.069	78.6
3	2.091±0.163	1.685±0.113	0.574±0.077	0.375±0.049	0.384±0.050	78.6

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.

SSR 195, accounting for 31.2% of all markers. However, there were no low-informative markers (≤ 0.25).

In the polymorphism analysis, the allele fragment sizes observed for proso millet genotypes ranged from 130 to 1100 bp. Each marker sustained analysis for genetic polymorphism, considering the number of alleles and polymorphism information content (PIC) value. For the loci with three or more alleles, the PIC values were predominantly higher than for other markers (0.359–0.595). The largest group included moderately informative markers ($0.5 > \text{PIC} > 0.25$), comprising 62.5% of all markers, and was capable of providing sufficient data on locus polymorphism.

In Group 1 genotypes of proso millet, the allele frequency varied from 0.012 to 0.916, with an average value of 0.379. In Group 2, excluding monomorphic markers, the minimum and maximum values were 0.061

and 0.939, with an average of 0.450. For Group 3, the values of the allele frequency ranged from 0.1 to 0.9, with a mean value of 0.455. The average allele frequency for all groups was 0.423. The microsatellite loci markers selected for use in this research reached analysis for variability of codominant data. Based on the analysis of allele distribution across the different genotype groups, the study determined various indicators. These comprised the number of observed alleles (Na), the effective number of alleles (Ne), the Shannon information index (I), the expected heterozygosity (He), the objective expected heterozygosity (uHe), and the percentage of polymorphic loci (P) (Table 3).

The allelic distribution analysis for codominant data showed that Group 1 gave the higher values for all the aforementioned values compared with the other groups, while nonsignificant differences resulted between

Table 4. AMOVA test of the studied groups in proso millet.

Source	d.f.	SS	MS	Est. Var.	%
Among Groups	2	46.60	23.30	0.21	9.0
Among Individuals	149	643.41	4.318	2.15	91.0
Within Individuals	152	0.00	0.00	0.00	0.0
Total	303	690.01		2.37	100
Stat	Value	P (rand >= data)			
Fst	0.091	0.001			

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

Groups 2 and 3. The higher values for these indices in Group 1 revealed the highest genetic variability of the proso millet genotypes. Allele frequencies also differed across the genotype groups. Of the 16 SSR markers evaluated, 78.6% alleles were polymorphic, while 24.4% alleles were monomorphic.

Overall, the number of alleles ranged from 1 to 4, depending on the marker. The primers SSR 458 and SSR 409 exhibited the highest number of detectable alleles, as well as the most polymorphic bands. Among the analyzed markers, the majority of the markers showed the highest polymorphism (100%), except SSR 460, SSR 404, and SSR 386. The polymorphism information content (PIC) values ranged from 0.261 (SSR 384) to 0.595 (SSR 409). The average PIC value was moderate, averaging 0.276 across all loci and 0.401 for polymorphic loci. Molecular genetic analysis identified that the primer SSR 409 was a highly informative marker ($PIC \geq 0.5$), characterized by four polymorphic alleles and a PIC value of 0.595.

Analysis of variance

The study quantified the distribution of genetic diversity within and between the genotype groups through the conduct of an AMOVA (Analysis of Molecular Variance) to identify genetic differentiation (Table 4). AMOVA revealed 9% of the total genetic variation was ascribable to differences among the genotype yield groups, while 91% was notable within groups. The fixation index ($F_{st} = 0.091$, $p = 0.001$) revealed the moderate but significant genetic differentiation among the low-,

medium-, and high-yielding accessions of proso millet. AMOVA disclosed some genetic diversity among the studied proso millet germplasm, indicating its suitability for breeding programs targeting improvement in seed size and grain yield.

Multivariate analysis of genetic and agronomic variations

Correlation analysis between the SSR markers (microsatellites) and valuable agronomic traits enables the early identification of promising genotypes with the highest grain yield, accelerating the development of new cultivars of proso millet. Generation of a heatmap was successful with the correlation analysis between 11 relevant SSR markers and agronomic traits (Figure 1). Correlation analysis revealed the two SSR markers (SSR 394 and SSR 448) showed no association with any of the agronomic traits. The four SSR markers (SSR 357, SSR 203, SSR 384, and SSR 430) also showed the lowest correlation with panicle length ($r = -0.170$, 0.179), general yield ($r = 0.203$), 1000-seed weight ($r = 0.180$), and productive tillering ($r = -0.188$).

Two markers, SSR 420 and SSR 365, showed the lowest negative relationship with general yield ($r = -0.171$ and $r = -0.177$), as well as a moderate to robust relationship with productive tillering ($r = 0.226$ and $r = -0.284$). The primer SSR 331 proved the most informative, as it revealed the least correlation with the traits of seed weight per panicle ($r = -0.163$), the number of seeds per panicle ($r = -0.166$), and plant stand survival to harvest ($r = 0.166$). Likewise, it exhibited a moderate



Figure 1. Heatmap of correlation analysis between SSR markers and agronomic traits in proso millet. NSPP – number of seeds per panicle, SWPP – seed weight per panicle, SWPA – number of seeds per plant, NSPA – seed weight per plant, TSW – 1000-seed weight, GY – general yield, PH – plant height, PL – panicle length, FLS – flag leaf surface, NI – number of internodes, SD – straw diameter, and PT – productive tillering.

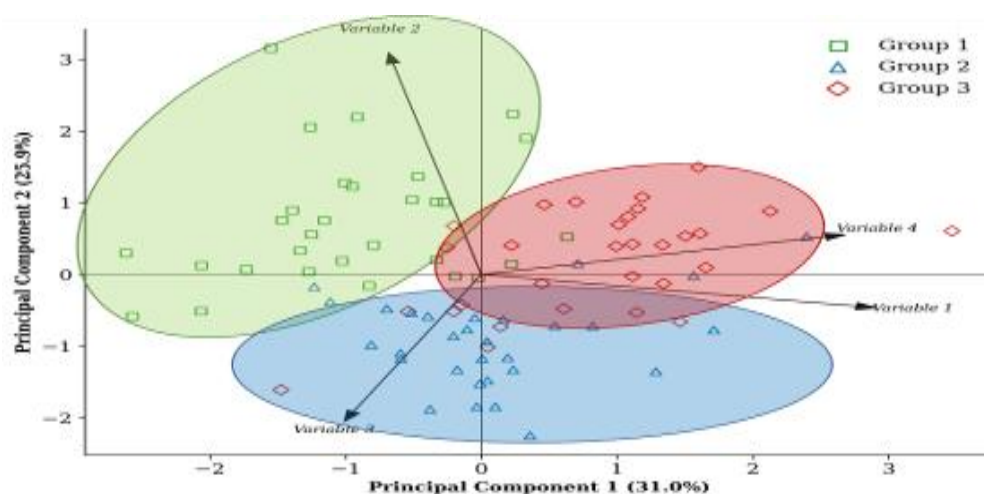


Figure 2. Distribution diagram based on principal component analysis in proso millet.

correlation with the number of internodes ($r = -0.208$) and a robust correlation with productive tillering ($r = 0.268$).

The primer SSR 409 gave varying degrees of correlation with some traits under consideration, and the most significant was being with the flag leaf area ($r = 0.389$). Moderate correlations appeared between the markers and the traits of flag leaf width ($-r = 0.237$; SSR 458), the number of internodes ($r = -0.208$; SSR 331), and productive tillering ($r = 0.226$; SSR 420). However, the highest positive correlation resulted between the flag

leaf surface and SSR 409 ($r = 0.389$); maximum negative correlation occurred between the productive tillering and SSR 365 ($r = -0.284$). The identified association with the molecular markers enunciated the potential for their use in further selection programs.

Based on the DNA polymorphism data of the studied genotypes of proso millet, enabled the principal coordinate analysis (PCoA). The analysis clearly separated low-yielding genotypes from medium- and high-yielding genotypes of the proso millet (Figure 2). The PCoA showed a tendency for separation

according to yield level. The placement of low-yielding accessions was primarily on the negative side of the first coordinate axis, whereas the high-yielding genotypes tended to cluster on the positive side. However, the substantial overlap among the genotype groups suggested moderate genetic differentiation rather than the presence of apparently distinct genetic pools. The set of markers used, although unable to clearly distinguish the second and third groups of the genotypes, exhibited a successful separation from the first group.

DISCUSSION

In proso millet (*P. miliaceum* L.), the valuable agronomic traits that played a significant role in its genetic improvement and yield formation include plant height, panicle length, 1000-grain weight, and the number and weight of grains per panicle and plant (Rajput and Santra, 2016; Zeinullina *et al.*, 2023). The 152 studied accessions of the proso millet succeeded in being grouped into three based on their productivity. The considerably higher grain yield was remarkable in Group 3 genotypes, despite lower values of the traits of productive tillering, seeds per panicle, seed weight per panicle, and 1000-seed weight. This can be due to the higher plant stand survival to harvest. The proso millet genotypes in Group 3 produced fewer seeds; however, they demonstrated greater vigor for plant stand survival until harvest and maintained the highest productivity through increased plant and panicle counts. In this case, although traditional yield components, such as 1000-seed weight, seeds per panicle, and seed weight per panicle, were relatively low, the plant stand survival to harvest appears to play a crucial role in determining overall grain yield.

Polymorphism information content (PIC) reflects the levels of genetic diversity in the population. Proso millet traditionally shows a low molecular marker diversity because it is a self-pollinating plant; hence, assessing polymorphism helps to screen vast germplasm collections to identify distinct parental lines, expanding the use of genetically variable

material in plant breeding. In general, the SSR markers with PIC values below 0.25 could reveal the low polymorphism; those with PIC values between 0.25 and 0.5 exhibit the moderate polymorphism, while the markers with PIC values of 0.5 or higher seem highly polymorphic (Sharma *et al.*, 2021). In the presented study, the majority of the used markers exhibited moderate-to-high polymorphism, suggesting their potential effectiveness for the analysis of genetic diversity. Genetic variation underwent evaluation across the proso millet germplasm comprising 152 accessions, with Shannon diversity indices ranging from 0.531 to 0.661 and a mean of 0.588. This was lower than the values reported by Cho *et al.* (2010), Hunt *et al.* (2011), Liu *et al.* (2016 and 2017), and Yazdizadeh *et al.* (2020); however, it emerged higher than those reported in the recent studies by Lv *et al.* (2025).

Overall, the more alleles per marker often showed an association with a higher PIC value, which further enhanced the ability to distinguish the proso millet genotypes. In this study, the PIC values ranged from 0.261 to 0.595, with a mean of 0.276 across all loci and 0.401 for polymorphic loci. However, these values were slightly higher than those reported in previous studies (Hunt *et al.*, 2011; Zargar *et al.*, 2023) but lower than those reported by Lee *et al.* (2017). These findings may be because of the differences in experimental material and the SSR marker sequences. The slightly lower PIC values in integration with AMOVA results may reflect the effect of human selection on the analyzed cultivars, which can reduce the genetic variation.

Correlation analysis of various morphological and agronomic traits plays a crucial role in the selection and improvement of crop genetic resources. The present results enunciated some moderate and significant relationships between the genotypic traits and the studied markers. One of the most informative markers was SSR 409, which proved to have an association with flag leaf area. Other markers, such as SSR 365 and SSR 331, indicated association with productive tillering. However, this study did not find a significant effect on grain weight per panicle

and thousand-seed weight, which was evidently inconsistent with the findings of Guo et al. (2025); however, explaining this discrepancy can refer to the influence of plant stand survival to harvest.

The PCoA analysis revealed a moderate level of genetic differentiation among the studied accessions of proso millet and indicated that the applied markers set was unable to effectively separate genotypes Groups 2 and 3. However, it clearly distinguished Group 1, comprising low-yielding accessions. Therefore, the set of molecular markers used in this study may potentially be applicable to identify the low-yielding genotypes for elimination at the early stages of proso millet breeding.

CONCLUSIONS

Based on the field data of proso millet (*P. miliaceum* L.) germplasm obtained during 2024–2025, these divided the genotypes into three groups based on grain yield. The data served as the basis for an independent review of agronomical indicators to identify potentially significant genotypes. The study revealed low-to-moderate genetic variability in both the number of alleles per locus across the genotype groups and the genetic diversity index. Genetic variability detection was successful in 29 alleles of the 16 SSR markers. The SSR markers SSR 409, SSR 365, and SSR 331 revealed significant associations with the agronomic traits directly impacting the final grain yield, and the primer SSR 409 proved the most efficient with the maximum PIC value (0.595). This set of markers can be useful for the early identification of low-yielding genotypes, allowing their elimination at the initial steps of proso millet breeding programs.

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