

## Genetic diversity and agronomic performance of Slovenian landraces of proso millet (*Panicum miliaceum* L.)

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**Abstract:** Proso millet (*Panicum miliaceum* L.) has many favored nutritional and agronomic traits, which makes it appropriate for cultivation and consumption all around the world. Genomic resources for proso millet are still very limited but the set of genomic data is improving. In this study, we genotyped six Slovenian landraces of proso millet (*P. miliaceum* L.) along with one Slovene autochthonous cultivar, Sonček. The chosen set of 11 SSR markers showed that there is low overall heterozygosity (0.561) among Slovenian landraces of proso millet. However, we were able to determine distinct groups on the dendrogram for different landraces and the cultivar by using UPGMA clustering. The PCoA scatter plot showed dispersion of unique individuals. The SSR markers used proved to be efficient for assessing the genetic diversity of Slovenian landraces of proso millet. Furthermore, we performed a 3-year field experiment and determined grain yield (ranging from 1032 to 1667 kg ha<sup>-1</sup>) and yield stability using Kang's yield stability index (YS<sub>i</sub>). The morphology of panicles and grain was described as well.

**Key words:** *Panicum miliaceum* L., genetic diversity, SSR markers, grain yield stability, panicle and grain morphology

### 1. Introduction

*Panicum miliaceum* L., most commonly named proso millet and broomcorn millet, is a member of the small millets group, which together with *P. miliaceum* encompasses six cereal crops: foxtail millet (*Setaria italica* L. Beauv), kodo millet (*Paspalum scrobiculatum* Michx.), finger millet (*Eleusine coracana* (L.) Gaertn.), little millet (*Panicum sumatrense* Rothex. Roem. and Schultz), and barnyard millet (*Echinochloa* spp.) (Goron and Raizada, 2015). According to CGIAR (Consultative Group on International Agricultural Research; <http://www.cgiar.org/>), proso millet has a 30% share of global millet production. Proso millet is grown for the production of small seeds, which are used as animal fodder and for human consumption (Habiyaremye et al., 2017b). *P. miliaceum* is a minor cereal today in terms of global economic importance, yet it is a very important food source among some of the world's poorest sections, especially for people living in hot and dry areas in developing and under-developed countries (Wang et al., 2016).

Proso millet is one of the world's oldest cultivated cereals. It appeared as a staple crop in northern China 10,000 years ago (Lu et al., 2009), and later spread to other parts of the world, including Slovene territory where it was grown as early as 1000 BC by the Celts (Ačko, 2012).

Although Slovenia is a small mid-European country (≈20 000 km<sup>2</sup> and ≈2 million inhabitants), proso millet was an essential dish for five centuries from the Middle Ages, when Slovene farmers consumed millet porridge on a daily basis (Ačko, 2012).

Proso millet can be described by some outstanding useful characteristics. Regarding favored nutritional traits, protein content (12.5%) is the highest among all small millets, and even higher than in the major cereals, rice and wheat (Saha et al., 2016). Furthermore, proso millet is gluten-free, which makes it appropriate for gluten-intolerant people. A few reports have revealed the medicinal benefits of consuming proso millet, e.g., lowering cholesterol and phytate, inhibiting certain cancers, preventing heart and liver diseases, and managing liver dysfunctions and diabetes (Zhang et al., 2014).

Proso millet also has lots of favored agronomic traits. It belongs to the grain crops which have extremely low water requirements. The reason for its drought tolerance is its short growing season, being mature within 60–90 days (Baltensperger, 1996). In addition, it can grow well in different poor soils, even with minimal agronomic input (Sabir et al., 2011).

Landraces have huge economic value for local cultivation because of adaptation to the agro-

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environmental conditions of the region where they have evolved (Lister et al., 2010). Slovenian farmers are sowing mostly landraces of proso millet. A landrace refers to a dynamic population of a cultivated plant that has historical association with a specific location. Local farmers maintain landraces through regeneration of seed in their traditional farming systems, without using any methods for genetic improvement (Camacho et al., 2005). One of the most important attributes for identifying landraces, also in autogamous species like proso millet, is genetic diversity (Zeven, 1998), which is massively utilized for crop improvement in the development of new cultivars, particularly when developing cultivars for marginal environments (dry, sandy, and acidic soils and hot climates) (Frankel et al., 1998). In such areas, proso millet (and most other small millets) but no other cereals can be productive (Changmei and Dorothy, 2014). Nowadays, the use of landraces of many crops is declining (Negri et al., 2009). Wide genetic erosion is caused by the employment of modern cultivars and hybrids (Camacho et al., 2005) or by a decrease in crop cultivation, which could in future result in narrow crop genetic resources and genetic diversity with the risk of extinction of some landraces, populations, and ecotypes (Saha et al., 2016). Thus, the preservation of landraces (and of other forms of ancient germplasm) has become necessary to enlarge the genetic basis of crop genomes. Preservation of landrace germplasm means that prior to seed storage in gene banks, landraces need to be agro-morphologically and genetically characterized. For the latter, molecular tools are used to further study the genetic diversity.

Compared to wheat, barley, and potatoes; proso millet is an underutilized crop. Therefore, research into its genetics, genomics, and breeding has been limited (Habiaryemye et al., 2017b). Furthermore, genetic analysis of proso millet is also difficult because of its polyploid nature (proso is allopolyploid;  $2n = 4x = 36$ ) (Li et al., 2012). However, this crop has received more research attention lately, and availability of genomic information is increasing with access to different genomic resources. Various molecular genetic markers, e.g., CAPS, RAPD, ISSR, AFLP, and SRAP (Habiaryemye et al., 2017b), have been used for the last two decades in order to estimate genetic relatedness in *P. miliaceum*. SSRs (simple sequence repeats) are the most widely used type of marker in genetic structure, genetic diversity, and genetic mapping of *P. miliaceum*.

In this study, genetic diversity among one proso millet cultivar and six landraces, still cultivated in eastern Slovenia, was studied using an SSR marker system. The yield of grain and its stability were determined in a 3-year field experiment. Panicle and grain morphological characteristics were also determined. The objectives of this study were (i) to genetically characterize Slovenian

landraces of proso millet, (ii) to prove the wide usefulness and high discriminating power of the SSR marker system for proso millet, and (iii) to analyze yield performance and yield stability of Slovenian proso millet landraces and elucidate a superior genotype (landrace) with the highest and most stable yield, for use in a possible breeding program in the future.

## 2. Materials and methods

### 2.1. Plant material

Six landraces of proso millet were collected in 2012 from farms where the owner claimed that they had autochthonous landraces of proso millet which had passed from generation to generation (Figure 1). Moreover, the Slovene autochthonous cultivar Sonček, which originates from the Gorenjska region, was added to the analyses. It is a drought- and lodging-resistant cultivar with high adaptability to growing in stress conditions with a lack of intensive fertilization (Ačko et al., 2012). In this study, the vernacular names of all landraces are used.

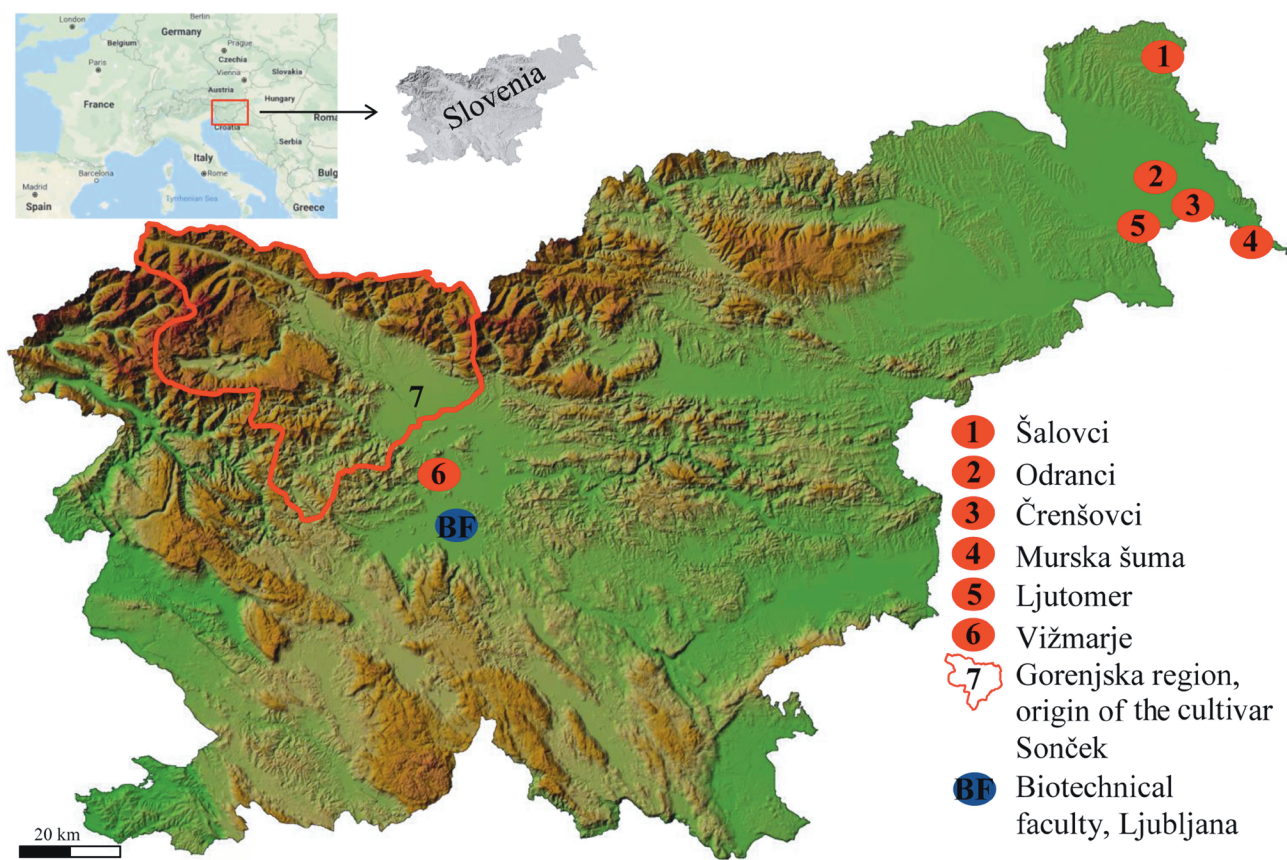
### 2.2. Genomic DNA isolation

Seeds of six landraces and the cultivar Sonček were sown into soil (Tonsubstrat, Klasmann, Germany) and incubated in a growth chamber at 25 °C with a 16 h light/8 h dark regime. Two weeks after sowing, eight individual plants were randomly chosen for SSR analysis, and DNA was extracted from a single plant using the modified CTAB method (Kump and Javornik, 1996). The DNA quality was visually checked using 0.8% agarose gel electrophoresis and quantified at 260 nm using a NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK). The final concentration of each DNA sample was adjusted to 20 ng  $\mu\text{L}^{-1}$ .

### 2.3. Microsatellite analysis

Cho et al. (2010) developed 25 microsatellite markers from the genomic DNA of proso millet. We tested 12 of them, namely GB-PaM-004, GB-PaM-013, GB-PaM-014, GB-PaM-023, GB-PaM-073, GB-PaM-085, GB-PaM-094, GB-PaM-106, GB-PaM-115, GB-PaM-121, GB-PaM-126, and GB-PaM-134, on four randomly chosen proso millet DNA samples. Only the GB-PaM-085 locus gave no banding pattern. Therefore, the other 11 loci were thereafter used in genetic analysis.

The PCR amplifications were performed in a final volume of 15  $\mu\text{L}$  containing 100 ng of genomic DNA, 1X PCR buffer (Promega), 2.0 mM  $\text{MgCl}_2$  (Promega), 0.2 mM of each dNTP (Sigma), 1.25 units of Taq DNA polymerase (Promega), 0.2  $\mu\text{M}$  of sequence-specific reverse primer, 0.2  $\mu\text{M}$  of forward primer with an M13(-21) tail, and 0.25  $\mu\text{M}$  of fluorescence-labelled universal M13(-21) primer (Applied Biosystems, Waltham, MA, USA). The forward primer of each pair was tailed with an M13 sequence (5'-TGT AAA ACG ACG GCC AGT-3'). The universal M13



**Figure 1.** Collection sites of Slovenian landraces of proso millet.

(-21) primer was labelled with 6FAM, VIC, NED, and PET, allowing detection of fluorescence.

The PCR amplifications were carried out with a 2720 Thermal Cycler (Applied Biosystems) with the following steps: hot start for 5 min at 94 °C, followed by five cycles at 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 1.5 min; continued with 35 cycles at 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 1.5 min. Reactions were completed by incubation at 72 °C for 8 min. Subsequently, the PCR products were diluted in formamide and subjected to capillary electrophoresis with an ABI 3130XL Genetic Analyzer. LIZ600 was adopted as the molecular weight standard.

#### 2.4. Field trials and phenotypic characterization

The field experiment was conducted on the experimental field at the Biotechnical faculty in Ljubljana, Slovenia, for four consecutive years (2013–2016). In 2014, excessive precipitation in August (205 mm) and September (204 mm) caused water retention on the soil surface for a longer period of time (a few days). Flood conditions in September prevented normal ripening of the proso millet plants, which were deformed, and the majority of grain dropped off. Therefore, we were unable to perform the harvest and characterize the grain yield in that year.

The experimental field has medium-deep silty-clay soil. The pH was 6.0–6.5 and the levels of  $P_2O_5$  and  $K_2O$  are in the optimum range (13–25 mg of  $P_2O_5$  per 100 g of soil, and 20–30 mg of  $K_2O$  per 100 g of soil). No chemical analysis of soil was performed during field trials.

The experimental setup was a randomized complete block design with three replications. Plot sizes were 5.6 m<sup>2</sup> in 2013 and 6.2 m<sup>2</sup> in 2015 and 2016. Plots were sown with a Wintersteiger plot seeder at a row space of 12.5 cm at the beginning of the July; harvest was carried out at the end of September/beginning of October (4th of July and 3rd of October in 2013, 8th of July and 30th of September in 2015, and 1st of July and 6th of October in 2016). The precrop was potato in 2013 and 2015 and soybean in 2016. Sowing density was 370 viable seeds m<sup>-2</sup>. Plots were manually weeded as required, and no fertilizers were added. Harvest was carried out at phenological stage 89 on the BBCH scale (fully ripe seed). The total yield for landraces and cultivar is given in terms of dry weight (kg ha<sup>-1</sup>). Descriptive morpho-agronomic traits of panicles (compactness and shape) and grain (color) were determined as per the descriptors for *P. miliaceum* and *P. sumatrense* (IBPGR, 1985). Weather conditions for all years are given in Figure S1.

## 2.5. SSR marker data analysis

The amplified alleles were identified and sized using Peak Scanner Software (v. 1.0; Applied Biosystems, Foster City, CA, USA). The data obtained were used as input for several data analyzing programs, according to their specific requirements.

Perfect synonyms were identified (Cervus) and removed, and a dataset of 28 genotypes showing unique DNA profiles was used to calculate basic statistics such as the number of alleles observed ( $N_A$ ), the values for observed, and expected heterozygosity ( $H_O$  and  $H_E$ ), polymorphic information content (PIC), frequency of null alleles ( $F_{null}$ ), and the probability of finding two identical genotypes (PI), using Cervus 3.0 software (Kalinowski et al., 2007).

Genetic variation among the six landraces and one cultivar was characterized in terms of number of alleles ( $N_A$ ), number of effective alleles ( $N_E$ ), Shannon's information index (I), expected heterozygosity or genetic diversity within a genotype ( $H_E$ ), observed heterozygosity for a single locus within a genotype ( $H_O$ ) and fixation index (F) using the genetic analysis package GenAEx v. 6.502 (Peakall and Smouse, 2006). To visualize differences between the sampled landraces and cultivar, a similarity matrix was used to run principal coordinates analysis (PCoA) (GenAEx v. 6.502). Analysis of molecular variance (AMOVA) with estimation of some F-statistics (Wright, 1965) was used to determine fixation index ( $F_{st}$ ),  $F'_{st}$  (standardized  $F_{st}$ ) and estimates of heterozygosity within genotypes ( $F_{is}$ ) according to Wright (1978).

Nei's coefficient (Nei et al., 1983) was used to estimate pairwise genetic distances for phylogenetic relationships among genotypes, and cluster analysis was performed according to the UPGMA (Unweighted Pair-Group Method with Arithmetic Averages) algorithm. Finally, an unrooted dendrogram from a distance matrix was generated using the program Darwin version 6 (Perrier et al., 2003).

## 2.6. Yield performance and yield stability analysis

The data for grain yield across 3 years were first subjected to combined analysis of variance (ANOVA). Year, which was taken as a factor, genotype and year  $\times$  genotype interaction were considered to be fixed effects and determined significant if  $P \leq 0.05$ . Replications were considered to be random effects.

Kang's yield stability index ( $YS_i$ ) was used to determine yield stability. Our main goal was to screen the performance potential of landraces, which could be used for further selection. Therefore, we used only one location and took it as a factor of environment to meet the requirements of genotype  $\times$  environment interaction in calculations of yield stability index. This approach was justified since year  $\times$  genotype interaction was highly significant (see

Results). We performed a 4-year field experiment but, unfortunately, in one year (2014), there was no grain yield due to unfavorable weather conditions; therefore, we determined yield stability based on data from 3 years. All data were analyzed using the packages nlme and agricolae in R software version 3.2.5 (R Core Team, 2016).

## 3. Results

### 3.1. Genetic diversity based on SSR markers

Out of 11 the SSR markers used in the analysis, 10 showed to be polymorphic and one monomorphic (GB-PaM-014). Polymorphic SSRs were used further to calculate several indices of genetic diversity. Identity analysis revealed 28 unique genotypes from the 56 proso millet samples analyzed.

A total of 34 alleles were detected, with an average of 3.4 alleles per locus. The loci GB-PaM-013, GB-PaM-094, and GB-PaM-121 had only two alleles, thus being the less informative ones. Locus GB-PaM-126 generated the highest number of alleles (six). Within genotypes, the expected heterozygosity ( $H_E$ ) for different loci ranged from 0.383 to 0.771 (mean = 0.561), while observed heterozygosity ( $H_O$ ) ranged from 0.000 to 1.000 (mean = 0.300).  $H_O$ , which represents the number of heterozygous individuals per locus, had a lower value than  $H_E$  at GB-PaM-023, GB-PaM-073, GB-PaM-106, GB-PaM-115, GB-PaM-121, GB-PaM-126 and GB-PaM-134 loci. This deficiency in  $H_O$  may be related to the presence of null alleles, whose frequency values were positive at GB-PaM-023, GB-PaM-073, and GB-PaM-126 for these loci. Four additional loci showed null allele frequencies not different from zero (GB-PaM-106, GB-PaM-115, GB-PaM-121, and GB-PaM-134). This result suggests that in these cases there are nonamplifying alleles. For the remaining three loci (GB-PaM-004, GB-PaM-013, and GB-PaM-094), no differences in the heterozygous deficit were observed. PIC values ranged from 0.353 to 0.726 with the average being 0.482. PI calculated for each locus ranged from 0.092 to 0.412 at GB-PaM-126 and GB-PaM-106, respectively, with an accumulated probability of identical genotypes for all loci of  $1.27 \times 10^{-6}$  (Table 1).

Descriptive statistics for the amount of genetic diversity found across plant genotypes are shown in Table 2. Shannon's information index (I) ranged from 0.189 to 0.658, with an average of 0.479.  $H_E$  for genotypes ranged from 0.136 to 0.434 (mean = 0.327), and  $H_O$  was 0.273. For fixation index (F), values were from -1.000 to 0.400 (mean = 0.214). Furthermore, F-statistics parameters for all genotypes together are shown in Table 2. AMOVA indicates that 97% of the observed variance was a result of variation between genotypes, and 3% was variance among landraces.

Genetic associations among the six landraces and one cultivar, representing 28 unique genotypes were

**Table 1.** Microsatellite loci used in this study and their genetic parameters.

Locus	Repeat motif	N <sub>A</sub>	H <sub>O</sub>	H <sub>E</sub>	PIC	F(Null)	PI
GB-PaM-004	(TG)8-(GA)9	3	1.000	0.597	0.508	-0.284	0.250
GB-PaM-013	(TCG)8	2	1.000	0.505	0.375	-0.333	0.375
GB-PaM-014	(CGT)3(CAT)(CGT)5						
GB-PaM-023	(GA)19	4	0.000	0.724	0.665	1.000	0.132
GB-PaM-073	(TC)21, (CGTG)4	4	0.000	0.647	0.572	1.000	0.198
GB-PaM-094	(AT)4, (GCG)4	2	1.000	0.505	0.375	-0.333	0.375
GB-PaM-106	(TC)19	4	0.000	0.383	0.353	0	0.412
GB-PaM-115	(AG)15	3	0.000	0.496	0.423	0	0.327
GB-PaM-121	(AT)7- (GTAT)9	2	0.000	0.463	0.354	0	0.398
GB-PaM-126	(GAA)5-(GA)20	6	0.000	0.771	0.726	1.00	0.092
GB-PaM-134	(AG)22	4	0.000	0.519	0.470	0	0.280
Mean		3.4	0.300	0.561	0.482	0.604	1.27 × 10 <sup>-6</sup>

N<sub>A</sub> – number of alleles observed; H<sub>O</sub> – observed heterozygosity; H<sub>E</sub> – expected heterozygosity; PIC – polymorphic information content; F(Null) – null allele frequency; PI – probability of identity.

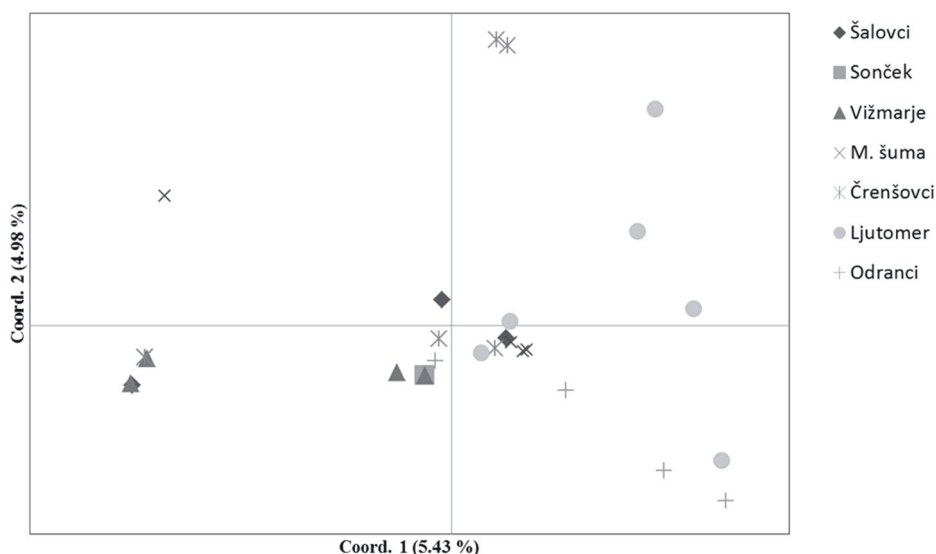
**Table 2.** Descriptive statistics of genetic diversity (mean and standard error) and F-statistics calculated across six landraces and one cultivar.

Genotype	N <sub>A</sub>	N <sub>E</sub>	I	H <sub>O</sub>	H <sub>E</sub>	F
Šalovci	1.727 ± 0.141	1.655 ± 0.120	0.428 ± 0.094	0.273 ± 0.141	0.343 ± 0.067	0.250 ± 0.312
Sonček	1.273 ± 0.141	1.273 ± 0.141	0.189 ± 0.098	0.273 ± 0.141	0.136 ± 0.070	/(*)
Vižmarje	1.909 ± 0.091	1.727 ± 0.091	0.572 ± 0.060	0.273 ± 0.141	0.399 ± 0.043	0.400 ± 0.291
M. šuma	1.636 ± 0.203	1.515 ± 0.164	0.380 ± 0.114	0.273 ± 0.141	0.259 ± 0.077	0.053 ± 0.315
Črenšovci	2.182 ± 0.263	1.888 ± 0.177	0.640 ± 0.112	0.273 ± 0.141	0.413 ± 0.066	0.333 ± 0.302
Ljutomer	2.182 ± 0.182	1.869 ± 0.120	0.658 ± 0.079	0.273 ± 0.141	0.434 ± 0.050	0.400 ± 0.291
Odranci	1.636 ± 0.152	1.600 ± 0.148	0.429 ± 0.103	0.273 ± 0.141	0.307 ± 0.074	0.143 ± 0.322
Total	1.792 ± 0.072	1.647 ± 0.056	0.479 ± 0.039	0.273 ± 0.051	0.327 ± 0.026	0.214 ± 0.112
<b>F-statistics</b>	<b>F<sub>is</sub></b>		<b>F<sub>st</sub></b>	<b>F<sub>st</sub> max</b>		<b>F<sub>st</sub>'</b>
<b>All genotypes</b>	0.716		0.028	0.038		0.727

N<sub>A</sub> – no. of different alleles per locus; N<sub>E</sub> – no. of effective alleles; I – Shannon's information index; H<sub>O</sub> – observed heterozygosity; H<sub>E</sub> – expected heterozygosity; F – fixation index; F<sub>is</sub>, F<sub>st</sub> – inbreeding coefficients; F<sub>st</sub>' – standardized F<sub>st</sub>; (\*) not observed as there is a single genotype.

investigated using PCoA. The analysis resulted in a total variation of 15.02%; the first and second principal coordinates explained 5.43% and 4.98% of genetic variation, respectively (Figure 2). The PCoA scatter plot showed that samples of proso millet landraces are much dispersed and no clear differentiation among group was observed. The exception is cultivar Sonček showing one genotype for all 8 different samples, which was expected and proved past breeding efforts in terms of some genetic stabilization. Despite high genetic diversity obtained within

the landraces, we were able to determine seven distinct groups on the dendrogram by using UPGMA clustering, calculations of which were based on the symmetrical matrix of pairwise genetic similarity estimates (Figure 3A). Only the cultivar Sonček formed a uniform cluster even if it consisted of two distinct genotypes. The only difference between them was due to the missing data or null allele occurrence at one locus. The grouping of the landrace Šalovci was also coherent, except one individual which did not cluster in the same group. On the contrary,



**Figure 2.** Scatter plot of principal coordinates analysis (PCoA) of six landraces and one cultivar of proso millet based on unique genotypes. PCo1 and PCo2 jointly accounted for 10.41% of the genetic variation observed.

the landrace Črenšovci turned out to be the most diverse, since there were six unique genotypes of which two were allocated to two other groups. Landrace Ljutomer was also a very heterogenic group, with six unique genotypes.

Based on a pairwise population matrix of Nei's genetic distances, the highest genetic distance was observed between landraces Vižmarje and M. šuma (0.645), and the lowest between Odranci and M. šuma (0.146). The landrace Vižmarje has the highest genetic distance of all the other landraces and the cultivar (0.431). On the other hand, the landrace Odranci has the lowest genetic distance (0.335) (Table 3).

### 3.2. Agro-morphological characteristics and yield stability indices

ANOVA for grain yield ( $\text{kg ha}^{-1}$ ) of the six landraces and one cultivar of proso millet tested for 3 years showed a significant difference ( $P < 0.05$ ) for year (Y), and a highly significant difference ( $P < 0.001$ ) for genotype (G) and for the interaction  $Y \times G$ .

Regarding landraces, the highest average 3-year grain yield was achieved by the landrace Črenšovci ( $1667 \text{ kg ha}^{-1}$ ), and the lowest was observed for the landrace Ljutomer ( $1281 \text{ kg ha}^{-1}$ ). Average grain yield of cultivar Sonček was  $1471 \text{ kg ha}^{-1}$  (Table 4).

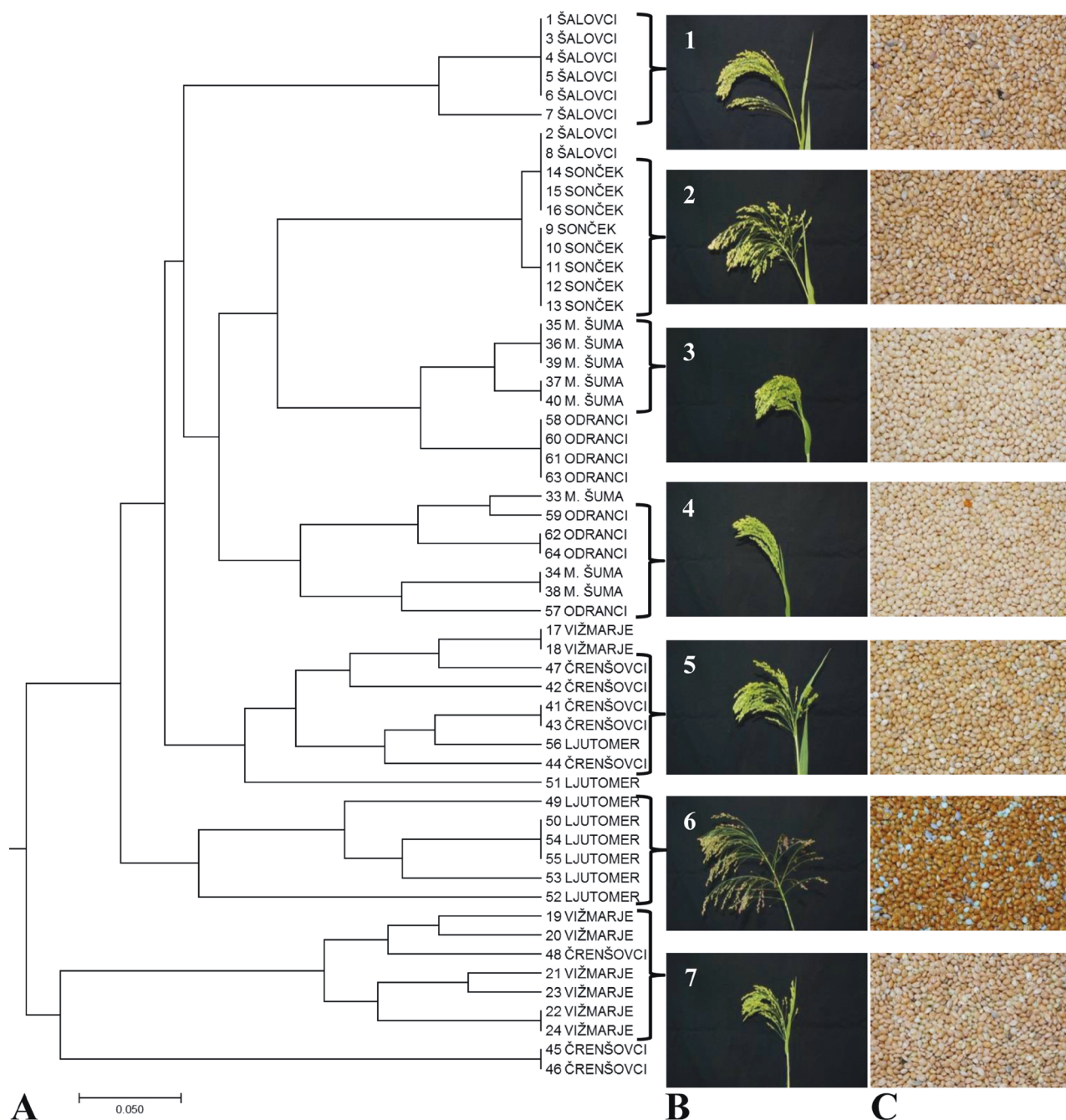
Genotype  $\times$  year interaction for grain yield was highly significant; therefore, the location for each year was taken as an independent environment in order to calculate stability statistics and to determine the stability of each genotype over the three environments. Nonparametric Kang's yield stability index ( $YS_i$ ), which simultaneously uses both mean yield and Shukla's variance ( $\sigma_i^2$ ) as

selection criteria showed that the landraces Črenšovci and Vižmarje, followed by the cultivar Sonček, were identified as the most stable genotypes in this study (Table 4).

The shape of the inflorescences of most of the landraces was 'contractum', meaning arched branches. Cultivar Sonček inflorescences were 'elliptic' (globose), and those of landrace Ljutomer 'patentissimum' which means diffused. Regarding compactness of the inflorescences, we noticed intermediate compactness (contractum) for two landraces, open inflorescences (miliaceum) for three landraces, and compact inflorescences (glosum) for the landrace Ljutomer and cultivar Sonček. The color of grains ranged from white and yellowish brown to reddish brown (Figures 3B and 3C; Table 4).

### 4. Discussion

In our 3-year field experiment with proso millet, the average yields by six landraces and one cultivar (from  $1281$  to  $1667 \text{ kg ha}^{-1}$ ) are comparable with those of some previous studies, which reported grain yields of  $1000$  to  $4000 \text{ kg ha}^{-1}$  from field experiments (Agdag et al., 2001; Seghatoleslami et al., 2008; Ačko et al., 2012; Sikora et al., 2013; Zhang et al., 2016; Caruso et al., 2018). Sometimes, yields are much higher, e.g.,  $8100 \text{ kg ha}^{-1}$  (Habiyaemye et al., 2017a), but in scientific research, it very much depends on the accessions/varieties, environmental conditions and experimental characteristics chosen, e.g., plot size, time and mode of harvest, agro-technique (irrigation, fertilization). Furthermore, yield stability governs the production efficiency of varieties and should be considered in breeding programs as well. In the 3-year field experiment, Kang's



**Figure 3.** A-Dendrogram of genetic relationships among six landraces and one cultivar of proso millet, generated with Nei's coefficient (Nei et al., 1983) and UPGMA cluster analysis; B-shape of the inflorescences of six landraces and one cultivar of proso millet; numbers represent recognized clusters: 1 – Šalovci, 2 – Sonček, 3 – Murska šuma, 4 – Odranci, 5 – Črenšovci, 6 – Ljutomer, 7 – Vižmarje; C-grains of six landraces and one cultivar of proso millet.

yield stability index (YS) which takes simultaneously mean yield and stability as selection criteria, landrace Črenšovci (average yield 1667 kg ha<sup>-1</sup>) turned out to be the superior genotype in this study, followed by landrace Vižmarje and cultivar Sonček. Intriguingly, landrace Črenšovci overcomes the cultivar Sonček in term of high yield and yield stability

meaning that it could be selected for future breeding efforts.

Worldwide cultivation and production of all millets has not increased or has even been declining in the last decade and a half (Dwivedi et al., 2012; Saha et al., 2016). Although proso millet has numerous agronomic and nutritional advantageous traits, its cultivation and production around

**Table 3.** Nei's coefficients of genetic distance.

	Šalovci	Sonček	Vižmarje	M. šuma	Črenšovci	Ljutomer	Odranci
Šalovci	0.000						
Sonček	0.295	0.000					
Vižmarje	0.423	0.381	0.000				
M. šuma	0.400	0.415	0.645	0.000			
Črenšovci	0.279	0.483	0.190	0.483	0.000		
Ljutomer	0.559	0.593	0.462	0.317	0.246	0.000	
Odranci	0.285	0.392	0.484	0.146	0.373	0.329	0.000
Average	0.373	0.426	0.431	0.401	0.342	0.418	0.335

**Table 4.** Average grain yield ( $\text{kg ha}^{-1}$ )  $\pm$  standard error, Kang's yield stability index ( $YS_i$ ), and descriptive morpho-agronomic traits of panicles and grains of six proso millet landraces and one cultivar.

Genotype	Average grain yield ( $\text{kg/ha}$ )	$YS_i^*$		Morpho-agronomic traits		
		Values	Selected genotypes	Inflorescence shape	Compactness of inflorescence	Color of fruit
Šalovci	1480 $\pm$ 281	-1		Contractum	Miliaceum	Yellowish brown
Sonček	1471 $\pm$ 152	3	+	Elliptic	Glosum	Yellowish brown
Vižmarje	1465 $\pm$ 195	4	+	Contractum	Miliaceum	Yellowish brown
M. šuma	1474 $\pm$ 190	-2		Contractum	Contractum	White
Črenšovci	1667 $\pm$ 237	9	+	Contractum	Miliaceum	Yellowish brown
Ljutomer	1032 $\pm$ 121	-10		Patentissimum	Glosum	Reddish brown and white
Odranci	1357 $\pm$ 255	-3		Contractum	Contractum	White
Mean		0				

\* Stable genotypes have a  $YS_i$  value greater than the mean  $YS_i$ .

the world are almost negligible compared to some other cereals. In 2016, production of small millet encompassed only 3.8% of that of wheat and rice, and an even smaller share (2.7%) of that of maize (FAO, 2018). One of the main reasons for decreasing proso millet production is its low grain yield (Dwivedi et al., 2012; Amadou et al., 2013; Goron and Raizada, 2015; Habiyaremye et al., 2017b). All small millets suffer from low grain yields ( $0.89 \text{ t ha}^{-1}$  in 2016) (FAO, 2018), and proso millet is no exception. Low grain yields can be attributed to a lack of scientific attention (Goron and Raizada, 2015). The only way to increase genetic yield potential is through breeding and selection of genotypes with high grain yields, which must be a major goal in any breeding program. To date, proso millet has been bred mainly through direct selection of promising germplasm, and conventional plant breeding (Habiyaremye et al., 2017b). However, a genetic linkage

map was constructed to allow the first QTL mapping study in proso millet (Rajput et al., 2016), thus posing an opportunity for marker-assisted selection and breeding for genetic improvement of complex traits such as yield, as has been successfully practiced, e.g., in barley (Schmierer et al., 2004), maize (Bouchez et al., 2002), wheat (Kuchel et al., 2005), and rice (Zhang et al., 1995). Landraces can be very good starting material for breeding, because they harbor genes that can improve existing plant varieties or introduce traits for countering biotic and abiotic stresses (Malik and Singh, 2006).

In order to reveal genetic distances among six Slovenian landraces and one cultivar of proso millet, 11 SSR markers (Cho et al., 2010) were used for genotyping. Ten out of 11 markers showed polymorphism and were therefore used for determination of genetic diversity. The average number of alleles per locus was 3.4 which is slightly lower than the



values obtained in some other studies performed on proso millet SSR genotyping: 4.9 (Hu et al., 2009), 4.4 (Cho et al., 2010), 4.9 (Hunt et al., 2011) and 12.9 (Rajput and Santra, 2016). In the studies listed, the number of accessions analyzed was high (from 50 to 118). On the other hand, Liu et al. (2016) and Hou et al. (2017) detected a lower number of alleles per locus (2.7 and 3.1, respectively), although they analyzed 88 and 56 accessions. However, sample size is one of the main factors that influences the number of alleles observed (Liao et al., 2014).

PIC value indicates the usefulness of an SSR locus for genotyping and germplasm evaluation. Our results showed that the selected microsatellites were moderately informative (average PIC = 0.482). Based on PIC value and another distinct discriminative index (PI), the locus GB-PaM-126 was shown to be the most informative out of the 10 loci analyzed. The overall low probability of obtaining identical genotypes ( $1.27 \times 10^{-6}$ ) indicated that the set of SSR markers used is effective for genotyping the sampled set of proso millet genotypes. The UPGMA dendrogram allowed the discrimination and characterization of genotypes into seven distinct clusters representing groups of landraces, with minor deviation of some genotypes from the major representative groups (Figure 3A). The most diverse groups as shown in the dendrogram are landraces Črenšovci and Ljutomer, both having six unique genotypes which clustered to different groups; the most homogeneous group was the cultivar Sonček. The results obtained show that there are genetic differences between landraces, although some landraces are not distinguishable from each other morphologically, e.g., landraces Črenšovci, Vižmarje, and Šalovci have similar inflorescences (shape and compactness) and grain color. Likewise, landraces M. šuma and Odranci are inseparable regarding the morphological traits of inflorescences and grains. On the other hand, cultivar Sonček and landrace Ljutomer have unique morphology, which distinguishes them from the others by these two characteristics (Figures 3B and 3C). The SSR markers used in our study were able to distinguish the majority of genotypes, which was not the case for some other published studies, e.g., that of Trivedi et al. (2015), who used 11 EST-SSR markers on 16 proso millet accessions but observed no diversity. However, the same study also used ISSR and SRAP markers which proved some allelic variation (Trivedi et al., 2015).

The overall heterozygosity values in our proso millet landraces and cultivar were low, thus implying the presence of narrow genetic diversity. The highly mixed genetic structure of the proso millet landraces might be a consequence of geographical distribution of their cultivation that shows possible interference of landraces not only because of the small distances between the places where they were grown but also because of the habits of farmers, who often exchange seeding material (Ačko,

2012). Besides that, Nei's genetic distances do not support geographical distance among populations, where for example landraces Odranci and M. šuma with the lowest value for genetic distance (0.146) are not in the closest geographic relation (Figure 1), although they have the same panicle (contractum) and grain (white) morphology. These results support the hypothesis that mixing of genetic material probably occurred in the past. Although some previous studies confirmed a positive correlation between geographic origin and genetic distance in proso millet (Hu et al., 2009; Hunt et al., 2011), it has been also demonstrated that genetic and geographical distance can have no correlation (Hou et al., 2017), as also turned out in our study. Intriguingly, Vižmarje and M. šuma, whose genetic distance was highest (0.645), turned out to also differ in inflorescence compactness (miliaceum vs. contractum) and grain color (yellowish brown vs. white).

The SSR-determined genetic diversity of the landraces and cultivar in this study was 0.327. For comparison, the genetic diversity of 118 (Hu et al., 2009) and 88 (Liu et al., 2016) proso millet accessions of Chinese germplasm, and 98 accessions from Europe and Asia (Hunt et al., 2011) was 0.834, 0.445 and 0.391, respectively. Furthermore, Rajput and Santra (2016) discovered a wide range of allelic diversity in a proso millet core collection of 90 genotypes in the USA, with 12.8 alleles per locus, showing that proso millet all around the world has not undergone a human-induced bottleneck and artificial selection, thus maintaining a high degree of heterozygosity.

In conclusion, the SSR marker set used in the present study proved to be effective for assessing genetic diversity and understanding the population structure of Slovenian landraces of proso millet. We discovered that there exists low overall heterozygosity among Slovenian landraces of proso millet, most likely caused by short geographical distance and similar pedo-climatic conditions. Proso millet grain is highly nutritious with positive effects on health, and the crop has many agronomic advantages. However, its cultivation is not widespread, mainly because of low yield which is a consequence of lack of genetic improvement. Proso millet is receiving attention in genetic research, and progress has been made thanks to the new genetic data obtained, e.g., gene maps, gene expression profiling, and NGS data, which will hopefully lead to greater exploitation of this crop. Landraces can be a very good source of genetic material for developing varieties, and genetic characterization is a first step towards new and improved varieties.

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