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Genetic diversity of common bean (*Phaseolus vulgaris* L.) germplasm from Serbia, as revealed by single sequence repeats (SSR)

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Abstract

Genetic diversity and structure of common bean (*Phaseolus vulgaris*) germplasm from Serbia, comprising 118 landraces and 18 cultivars, was assessed with the application of 27 Single Sequence Repeats (SSR) markers. Thirteen accessions from Agricultural Institute of Slovenia were used as references for gene pool determination. Main parameters of genetic diversity were calculated for each SSR loci, i.e. number of different and rare alleles, number of effective alleles, Shannon's information index, observed and expected heterozygosity and polymorphic information content. A total of 445 allelic variants, with 16.5 alleles per locus on average, were detected. Mean gene diversity (He= 0.79) indicated sufficient reservoir of genetic variation preserved in studied bean germplasm. Landraces displayed higher variability compared to cultivars (405 in relation to 233 allelic variants). Genetic

structure and relatedness of accessions was assessed by model-based method and hierarchical clustering method in combination with genetic distance calculation. The Bayesian clustering model implemented in STRUCTURE software, on the primary level (K=2), revealed clear separation of accessions into two groups, corresponding to gene pool affiliation. Mesoamerican gene pool (M) was represented with 23.5% of accessions, while Andean (A) was larger, composed of 68.4% of studied germplasm. Small group (8.1%) showed admixed genetic structure between two gene pools. Additional variation in respect to two recognized gene pools was revealed (K=3), whose basis was acknowledged to be within Andean gene pool. Further subdivision of accessions (K=8), mainly according to the seed forms, was observed. Genetic distance analysis associated with Neighbour-joining clustering method revealed grouping pattern of landraces and cultivars corresponding to the gene pool and their seed phenotypes. Classification and structuring of the bean accessions according to and beyond the gene pool of origin should facilitate conservation strategies and breeding of this material. Combining the information of phenotypic variation obtained in previous research and molecular data reveled in this study will assist in selection of parental components for breeding, or in the choice of smaller sample in order to further acknowledge their breeding value. In addition, obtained results of this work should serve as an additional information on common bean germplasm variation in Western Balkans and beyond, in Europe.

Keywords: *Phaseolus vulgaris*, SSR, genetic diversity, gene pool

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most valuable vegetable crops for human consumption since it is rich in proteins, fibres, vitamins, minerals and many other antioxidant compounds (Broughton et al., 2003; Maras et al., 2015; Sitohy et al., 2020). Being a diverse crop in terms of cultivation methods, use, phenotypic diversity and range of environments to which it is adapted, the common bean is grown worldwide (De Ron et al., 2016). In many European countries, *Phaseolus vulgaris* is a significant component of a traditional diet and life.

Phaseolus vulgaris has undergone two independent domestication events in primary centres of origin: one in Middle America, and one in Andes. As a result, two highly differentiated gene pools were formed: Mesoamerican and Andean, which are distinguished and recognized according to their phenotypic, biochemical and genotypic differences (Gepts et al., 1986; Gepts, 1999; Šuštar-Vozlič et al., 2006; Raggi et al., 2013; Carović-Stanko et al., 2017; Gioia et al., 2019; Savić et al., 2020). These two gene pools are also characterized by geographic and partial reproductive barriers (Gepts and Bliss, 1985; Gioia et al., 2013). Furthermore, within each gene pool, there is subdivision of this species to many eco-geographic races and seed forms (market classes). Inter-gene pool and interracial crosses of genotypes can exhibit negative combining ability and lethality problems, which aggravates the breeding of common bean (Singh et al., 1991; Kelly et al., 1998; Blair et al., 2007).

Gene pool affiliation has usually been determined based on variation of the main storage protein of the common bean, phaseolin (Gepts and Bliss, 1988; Šuštar-Vozlič et al., 2006; Logozzo et al., 2007; Carović-Stanko et al., 2017; Savić et al., 2020). Mesoamerican origin of common bean genotypes is associated with phaseolin types S (*Sanilac*), M (*Middle America*) and B (*Boyaca*), while genotypes with phaseolin types T (*Tendergreen*), C (*Contender*) and H (*Huevo de Huanchaco*) belong to the Andean gene pool. However, since Nani et al. (2011) identified three indel spanning markers SHP1-A, SHP1-B and SHP1-C, newer researches more rely on these marker systems for gene pool identification (Maras et al., 2015, 2016; Pipan and Meglič, 2019).

From primary centres of origin and domestication in America, common bean spread worldwide (Zeven, 1997; Maras et al., 2015). It is believed that the common bean arrived in Europe on two occasions; Mesoamerican beans through Spanish and Portuguese exploration of the Americas around 1506; and Andean beans somewhere later, in 1528, during Pizzaro's expeditions in Peru (Gioia et al., 2013). In Europe, common bean landraces and cultivars evolved under diverse environments, cropping systems and farmers preferences (Zeven, 1997; Carović-Stanko et al., 2017; Pipan and Meglič, 2019). In addition, outcrossing among Andean and Mesoamerican genotypes facilitated in the development of high genotypic and phenotypic diversity of the European common bean (Rodino et al., 2006; Gioia et al., 2013).

Angioi et al. (2010) and Gioia et al. (2013) focused their research on hybridization phenomena of Andean and Mesoamerican germplasm in Europe. The presence of hybrid genotypes in high proportion within the European germplasm was revealed with chloroplast microsatellites (cpSSRs) and two unlinked nuclear loci (phaseolin and PvSHP1). Hegay et al. (2013) and Sinkovič et al. (2019) observed signs of introgression on the phenotypic level. Zhang et al. (2008) believe that identification of putative hybrids is of special interest since these genotypes are a reflection of growing regions and are adapted to ecological conditions within that region.

In Serbia, people have grown common bean for centuries, establishing it as an important part of their diet and traditional life. Even though commercial cultivars have been developed and are widely represented in production, common bean landraces are still maintained and used by farmers and people in rural and marginal areas (Scarano et al., 2014; Mallor et al., 2018; Savić et al., 2020). In Europe (including Serbia) development of new cultivars was instituted with the aim to maintain phenotypes within each seed form (market class), usually by selecting parental components among elite material. This resulted in narrowing the genetic base of this elite germplasm, compromising long-term genetic gain (McClean and Lee, 2007; Gioia et al., 2019). In order to address these problems, breeders sought to incorporate new variability, most commonly by exploring the existing diversity found among landraces and unadapted germplasm.

Genetic collections of the common bean in Serbia, consisting of seeds of traditional and modern cultivars and landraces, are maintained within breeding institutes (Vasić et al., 2009). Knowledge of genetic diversity preserved in these collections is crucial for proper conservation, further research, selection of parental components and for defining breeding strategies. Landraces are described as genetically diverse material with traits specific for growing regions. They are traditionally grown in low input systems, adapted to local agro-climatic conditions and display a high level of phenotypic diversity. All of this makes landraces interesting material for conservation, research and implementation in breeding programs (Carović-Stanko et al., 2017; Gioia et al., 2019).

Selected set of landraces and commercial cultivars from Serbia have already been characterized for diversity on a phenotype level, by assessing morphological traits chosen according to international descriptors. In addition, gene pool of origin was identified based on variation of phaseolin types (for

more information see Savić et al., 2020). However, in order to better understand genetic variation and relationships among landraces and cultivars on a molecular level, complementary study on previous research was performed. Single sequence repeats (SSR) markers were chosen for the analysis, because they are abundant and widely distributed in the genome, codominantly inherited, highly polymorphic and repeatable (Yu et al., 1999; Maras et al., 2015; Pipan and Meglič, 2019). Therefore, the aim of this study was to: (i) assess the allelic diversity of the common bean germplasm from Serbia and determine relationships among the accessions, and (ii) investigate the genetic structure and organization of genetic diversity of the studied germplasm within and beyond gene pools of origin.

2. Material and Methods

2.1. Plant material

A total of 136 accessions from the Serbian common bean genetic collection maintained at the Institute of Field and Vegetable Crops, Novi Sad (IFVCNS) were analysed in this paper. This included 118 local landraces collected from 53 sites in Serbia (Supplementary Material 1) in timescale of 1970-2014 and 18 commercially available cultivars from Serbia: Rozalija (C1), Žutotrban (C2), Sremac (C3), Balkan (C4), Slavonski žutozeleni (C5), Pasuljica P-1 (C6), Biser (C7), Medijana (C8), Oplenac (C9), Panonski gradištanac (C10), Panonski tetovac (C11), Aster (C12), Poboljšani gradištanac (C13), Galeb (C14), Zlatko (C15), Dvadesetica (C16), Belko (C17) and Maksa (C18), Table 1. Part of the studied material was collected during field expeditions, while the rest was acquired via seed exchange with other institutions. Landraces and cultivars were classified according to seed traits in several forms, most commonly grown in Serbia: *Roseus* (pink seed colour), *Versicolor* (seed coat pattern), *Griseus* (greenish-yellow seed colour), *Aureus* (yellow and golden-yellow seed coat colour), *Albus* (white seed colour) and other (red, cream, brown, black seed colour).

As references for gene pool determination, accessions of familiar phaseolin type (type T – PHA131, PHA306, PHA309, PHA318, PHA336; type C – PHA181, PHA222, PHA29, PHA315, PHA390; type S – PHA245, PHA368, PHA371) from Agricultural Institute of Slovenia (AIS) were

included in the study (Supplementary Material 1). Phaseolin types T and C indicate germplasm origin from Andean, while the Mesoamerican gene pool is determined with type S. For detailed phenotypic characterization, see Savić et al. (2020).

2.2. SSR analysis

Plants were grown in greenhouse conditions until the phase of the first true leaves at Agricultural Institute of Slovenia. DNA was extracted from a total of 60 to 100 mg of bulked fresh plant tissue (4 plants per accession), using a BioSprint 15 DNA Plant Kit (Qaigen) on KingFisher (Thermo) isolation robot according to optimized manufacturer's instructions.

A set of 27 genome-specific SSR (single sequence repeats; microsatellites) markers distributed across all linkage groups was used for genetic diversity and genetic structure analysis of selected material (Supplementary Material 1). For the identification of genotypes gene pool affiliation (Mesoamerican/Andean) three indel spanning markers (SHP1-A, SHP1-B and SHP1-C) developed by Nanni *et al.* (2011) were used.

The final volume of PCR reaction was 11.5 µL, which included: 8.4 ng genomic DNA, 1 µL 10x PCR buffer (Biotools), 0.2 µL of each 10 mM dNTP (Sigma-Aldrich), 0.5 µL of 50mM $MgCl_2$ (Biotools), 0.1 µL of 10 µM forward primer (Sigma-Aldrich), 0.25 µL of 10 µM reverse primer (Sigma-Aldrich), 0.183 µL of 10 µM 5′-fluorescently labelled primer (6-FAM, NED or HEX; Omega), and 0.5 µL of 5 U Taq DNA polymerase (Biotools). The forward primer of each SSR had an added 18-bp tail sequence of 5′-TGTAAAACGACGGCCAGT-3′ (M13(− 21)).

PCR analyses were performed on a thermal cycler (Veriti, ThermoFisher Scientific) under touch-down conditions: 94°C for 4 min; 15 cycles at 94 °C for 1 min; decreased temperature from 60 (62) °C to 49.5 (51.5) °C at 0.7 °C per cycle for 30 s; 72 °C for 1 min; followed by 23 cycles at 94 °C for 30 s; 53 °C for 30 s; 72 °C for 1 min; and final extension for 5 min at 72 °C, as described by Pipan and Meglič (2019). PCR conditions were dependent on each primer pair. Fragment analysis was performed on a genetic analyser (3130XL; Applied Biosystems). Allele lengths were determined by

comparison with an internal size standard (GeneScan-350 ROX; Applied Biosystems) using the GeneMapper 4.0 software (Applied Biosystems).

Table 1. List of accessions from Serbian common bean genetic collection, used for genetic analysis

Accession	Type	Seed form	Accession	Type	Seed form
L1	III	other	**L56**	I	*Versicolor*
L2	III	*Aureus*	**L57**	I	*Griseus*
C1	I	*Roseus*	**L58**	I	*Albus*
L3	I	*Roseus*	**L59**	II	*Albus*
L4	I	*Roseus*	**L60**	I	*Griseus*
L5	I	*Roseus*	**C14**	I	*Albus*
L6	I	*Roseus*	**C15**	I	*Aureus*
L7	I	*Roseus*	**C16**	I	*Albus*
L8	I	*Roseus*	**C17**	I	*Albus*
L9	II	*Griseus*	**C18**	I	*Albus*
L10	I	*Griseus*	**L61**	I	*Griseus*
L11	II	*Albus*	**L62**	I	*Griseus*
L12	I	*Albus*	**L63**	I	*Griseus*
C2	I	*Versicolor*	**L64**	I	*Griseus*
L13	I	*Versicolor*	**L65**	I	*Aureus*
L14	I	*Versicolor*	**L66**	I	other
L15	I	*Versicolor*	**L67**	I	other
L16	I	*Versicolor*	**L68**	I	*Griseus*
L17	I	*Versicolor*	**L69**	I	*Griseus*
L18	I	*Versicolor*	**L70**	I	*Versicolor*
L19	I	*Versicolor*	**L71**	I	other
L20	I	*Griseus*	**L72**	I	other
L21	I	*Griseus*	**L73**	I	*Griseus*
L22	I	*Griseus*	**L74**	I	*Griseus*

L23	I	*Griseus*	**L75**	I	*Griseus*
L24	I	*Griseus*	**L76**	I	*Versicolor*
L25	I	*Griseus*	**L77**	I	*Albus*
L26	I	*Griseus*	**L78**	I	*Albus*
L27	I	*Griseus*	**L79**	III	*Albus*
L28	I	*Aureus*	**L80**	I	*Griseus*
L29	I	other	**L81**	I	*Griseus*
L30	I	other	**L82**	I	*Versicolor*
L31	I	*Griseus*	**L83**	I	*Albus*
L32	I	other	**L84**	I	*Griseus*
L33	I	other	**L85**	I	*Versicolor*
L34	I	*Griseus*	**L86**	I	*Griseus*
L35	I	other	**L87**	I	other
L36	I	other	**L88**	I	*Albus*
L37	I	*Griseus*	**L89**	III	*Albus*
L38	I	*Roseus*	**L90**	I	other
L39	I	*Roseus*	**L91**	I	*Albus*
L40	I	other	**L92**	II	*Aureus*
L41	I	other	**L93**	III	*Albus*
L42	I	*Roseus*	**L94**	II	other
L43	II	*Aureus*	**L95**	I	*Griseus*
L44	I	*Aureus*	**L96**	I	*Versicolor*
L45	I	*Aureus*	**L97**	I	other
L46	I	*Aureus*	**L98**	III	*Albus*
C3	I	*Griseus*	**L99**	I	*Versicolor*
C4	I	*Albus*	**L100**	I	other
C5	I	*Griseus*	**L101**	I	*Versicolor*
C6	II	*Albus*	**L102**	I	*Griseus*
C7	I	*Albus*	**L103**	I	*Griseus*
C8	II	*Albus*	**L104**	III	*Albus*

L47	I	*Albus*	**L105**	I	*Albus*
L48	I	*Albus*	**L106**	I	*Albus*
L49	I	*Albus*	**L107**	II	*Albus*
L50	I	*Griseus*	**L108**	I	*Versicolor*
L51	I	*Albus*	**L109**	I	*Albus*
C9	I	*Albus*	**L110**	I	*Versicolor*
L52	I	*Albus*	**L111**	I	*Versicolor*
L53	I	*Albus*	**L112**	I	*Albus*
C10	I	*Albus*	**L113**	I	*Albus*
C11	I	*Albus*	**L114**	I	*Griseus*
L54	II	*Aureus*	**L115**	I	*Versicolor*
C12	I	*Albus*	**L116**	III	*Albus*
C13	III	*Albus*	**L117**	I	*Albus*
L55	I	*Versicolor*	**L118**	I	*Albus*

Type – plant growth habit (I – determinate bush, II – indeterminate bush, III – indeterminate prostrate or vining)

2.3. Data analysis

For each SSR locus, main parameters of genetic diversity were calculated in GenAlEx 6.1 (Peakall and Smouse, 2006) and Microsatellite-Toolkit (Park, 2001) software. They included number of alleles (Na), number of alleles with frequency ≥ 5%, number of rare alleles, number of effective alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC).

STRUCTURE 2.3.3 software was employed to establish genetic structure of common bean collection. STRUCTURE uses Bayesian clustering approach, applying Markov Chain Monte Carlo (MCMC) algorithm, to study difference in accessions distribution among population by placing accessions into clusters that share similar variation patterns. Bayesian clustering approach is described by the posterior probability that each accession belong to each genetic cluster, while each cluster (K) is characterized by a subset of allelic frequencies identified in the data. Beside determining and assigning

accessions to genetic clusters, this method is used to identify admixed accessions by investigating hybridization zones of individuals from different clusters that give genetically recombined offspring (Pritchard *et al*., 2009). In the study of common bean collection from Serbia, the most probable number of clusters (Q value) was determined with ten independent runs for each K (1 to 10) for the admixture model, with burning period of 50.000 followed by 500.000 Markov Chain Monte Carlo repeats. MCMC algorithm initiates by randomly assigning accessions to a pre-determined number of clusters. After that, allelic frequencies are estimated for each cluster and accessions are re-assigned based on those frequency assessments. According to Evanno delta K statistics (Evanno et al., 2005), implemented in the software Structure Harvester (Earl and von Holdt, 2011), the real K value was determined based on the increase in the likelihood rations between the runs. An accession is assigned to a specific cluster when the percentage of membership was $Q \geq 80\%$, while the accessions with membership coefficient $Q < 80\%$ are believed to be of admixed origin (putative hybrids).

For more detailed analysis of genetic structure and relationships among genotypes, DARwin software (https://darwin.cirad.fr/) was applied to perform cluster analysis based on similarity matrix and construct dendrogram using Neighbour-joining method (NJ).

3. Results

The whole set of 27 SSR markers chosen for the diversity study of the Serbian common bean genetic collection proved to be polymorphic, producing more than one allelic variants. A total of 445 alleles were scored for the studied collection, which included cultivars, landraces and reference accessions. The average allele number per SSR was 16.5, ranging from six alleles for loci BM155, BM210 and BMd044 to 25 alleles for loci GATS91 and ATA002. The highest number of effective alleles (9.35) and the most alleles with frequency over >5% were found in locus BMd001. Total number of rare alleles was 303, which accounted for 68.1% of all alleles detected in the studied germplasm (Table 2).

Table 2. Parameters of genetic variability of Serbian common bean collection and reference cultivars

Locus	Na	Allele frequency ≥ 5%	Number of rare alleles	Ne	I	Ho	He	PIC
ATA003	19	6	13	6.87	2.26	0.74	0.85	0.84
ATA004	15	4	11	3.82	1.77	0.44	0.74	0.71
ATA005	20	6	14	6.60	2.28	0.93	0.85	0.83
ATA007	17	5	12	3.42	1.74	0.30	0.71	0.68
ATA016	12	5	7	4.41	1.78	0.91	0.77	0.74
GATS91	25	5	20	8.38	2.49	1.00	0.88	0.87
ATA002	25	5	20	5.31	2.09	0.85	0.81	0.79
BM172	22	6	16	8.43	2.45	0.92	0.88	0.87
BMd001	24	9	15	9.35	2.46	0.99	0.89	0.88
ATA020	21	5	16	6.52	2.27	0.65	0.85	0.83
Pv-ag004	21	5	16	5.80	2.14	0.94	0.83	0.81
ATA010	13	5	8	4.25	1.75	0.31	0.77	0.73
BM155	6	4	2	3.53	1.36	0.87	0.72	0.67
BM170	20	6	14	8.07	2.40	0.95	0.88	0.87
BM183	8	5	3	3.26	1.41	0.93	0.69	0.64
BM210	6	2	4	2.14	0.85	0.97	0.53	0.42
BMd044	6	4	2	2.69	1.23	0.45	0.63	0.58
ATA009	22	8	14	8.18	2.45	0.75	0.87	0.87
ATA145	20	3	17	3.68	1.80	0.31	0.73	0.69
GA16	13	6	7	6.12	2.04	0.68	0.84	0.82
ATA006	22	7	15	8.98	2.52	0.79	0.89	0.88
BM157	14	6	8	5.88	2.00	0.60	0.83	0.81
BMd042	18	4	14	6.08	2.18	0.98	0.84	0.82
ATA289	17	4	13	3.87	1.74	0.97	0.74	0.71
PvSHP1-A	13	6	7	5.52	1.93	0.95	0.82	0.79
PvSHP1-B	11	6	5	5.89	1.89	1.00	0.83	0.81
PvSHP1-C	15	5	10	5.45	2.03	0.92	0.82	0.79

average	**16.5**	**5.2**	**11.2**	**5.65**	**1.97**	**0.78**	**0.79**	**0.77**
total	**445**	**142**	**303**					

Na – number of alleles, Ne – number of effective alleles, I - Shannon's information index, Ho –observed heterozygosity, He - expected heterozygosity, PIC - polymorphic information content

Loci BMd001 and ATA006 generated the highest values of expected heterozygosity (0.89) and PIC (0.88). The locus ATA006 scored the highest value of Shannon's information index (2.58). Observed heterozygosity ranged from 0.30 (locus ATA007) to 1.00 (loci GATS and PvSHP1-B). Average values of Shannon's information index (1.97), expected heterozygosity (0.79) and PIC (0.77) indicated that all SSR markers showed sufficient polymorphism and are suitable for common bean diversity study.

Main genetic diversity parameters were calculated for each common bean form determined according to seed traits (Table 3). Overall, landraces and cultivars from the *Albus* group showed the greatest diversity for all parameters, except number of alleles with a frequency higher than 5%. On contrary, the lowest diversity was found among accessions from the *Rosues* group, with slightly larger expected heterozygosity (0.76). The highest average number of alleles with a frequency higher than 5% was observed in the *Aureus* form. Rare alleles were not found among common bean genotypes from *Roseus* and *Aureus* groups, while for the other groups ranged from 3 (*Versicolor*) to 4.96 (*Albus*). Gene diversity (He) ranged from 0.67 (*Griseus*) to 0.78 (*Albus*). Observed heterozygosity was the highest among accessions from other (0.82) and *Aureu*s (0.81) groups. Almost twice as many alleles were scored among landraces (405) compared to cultivars (233). In addition, the percentage of rare alleles was much larger in landraces (67.6%) in relation to cultivars (29.6%). Average values of all the other parameters of genetic diversity (number of effective alleles, Shannon's information index, observed and expected heterozygosity) were similar between the two groups.

Table 3. Genetic diversity calculated for 27 SSR loci considering six groups defined according to the seed form; cultivars and landraces separately

Forms	Total number of accessions	Na	Na average	Allele frequency ≥ 5%	Number of rare alleles	Ne	I	Ho	He
Roseus	10	144	5.33	5.33	0	3.39	1.33	0.66	0.76
Versicolor	21	205	7.59	4.59	3	4.22	1.57	0.72	0.76
Grisues	35	231	8.55	4.52	4.03	3.88	1.49	0.79	0.67
Aureus	10	175	6.48	6.48	0	4.33	1.56	0.81	0.73
Albus	42	294	10.89	5.93	4.96	5.17	1.84	0.77	0.78
other	18	242	8.96	5.06	3.52	5.06	1.76	0.82	0.76
cultivars	18	233	8.63	6.07	2.56	5.28	1.79	0.79	0.78
landraces	118	405	15.00	4.85	10.15	5.32	1.91	0.78	0.78

Na – number of alleles, Ne – number of effective alleles, I - Shannon's information index, Ho – observed heterozygosity, He - expected heterozygosity

According to the Bayesian clustering model implemented in STRUCTURE software, the most informative number of subgroups was two (K=2). The second largest peak of Delta K value was observed for three subgroups (K=3), while studied accessions also displayed classification at eight subgroups (K=8) (Fig. 1).

Affiliation to the gene pools was identified when maximum likelihood and Delta K values were two (K=2), assigning accession to Mesoamerican (M; marked red in Fig. 2a) or Andean (A; marked green) group. This was confirmed based on allocation of reference accessions for gene pool classification. Total of 91.9% genotypes had membership coefficients higher than 0.80, implying that the majority of samples were strongly assigned to the groups. In addition, 11 genotypes (8.1%) were with membership coefficients lower than 0.80, showing admixed genetic structure between two groups (gene pools) (Supplementary Material 1). First group (M) included 32 genotypes (23.5% of studied germplasm) and reference accessions with mainly Mesoamerican phaseolin type. It comprised 21 landraces and 11 cultivars. A majority of genotypes in group M were from the *Albus* seed form (84%) with primarily medium (56%) and large (34%) seed weight. Second group (A) comprised 93 genotypes

(68.4% of studied germplasm) and reference accessions with mainly Andean phaseolin types (Fig. 2). It included 88 landraces and five cultivars. Genotypes with coloured and medium to large seeds predominated in this group (*Griseus*, *Aureus*, *Roseus* and *Versicolor* forms). Only 13 genotypes within second group had white seed coats with large seed weight in general. Genotypes identified as potential hybrids between the gene pools (11 accessions) according to STRUCTURE analysis were primarily landraces (5 *Albus*, 3 *Aureus*, 1 *Grisues*, 1 *Versicolor* and 1 *other*) with phaseolin type T.

For K = 3 grouping pattern, further division of the Andean group into two additional subgroups was observed. In this scenario, 88.9% of genotypes had membership coefficient higher than 0.80, while the rest showed admixed origin between the three subgroups (11.1%). First subgroup, M (marked red in Fig. 2b), remained the same, comprising 23.5% of studied germplasm. Second subgroup, A1 (marked green), was composed of 45 landraces that belonged to mainly *Griseus*, *Roseus* and *Versicolor* forms, two cultivars (Oplenac and Aster) of large white seeds and six reference accessions. Third subgroup, A2 (marked blue), comprised 34 landraces of largely *Griseus* and *Versicolor* common bean forms, 4 cultivars (Rozalija, Žutotrban, Sremac and Slavonski žutozeleni) and three reference accessions.

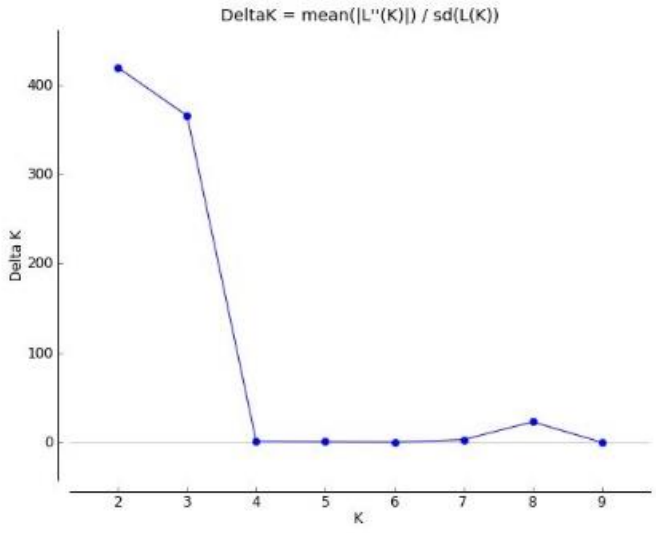

Figure 1. Estimation of most likely subgroups number according to Evanno's method (Evanno et al., 2005)

Another subdivision of a studied germplasm with classification criteria in which membership coefficient was higher than 0.50, corresponding to seed traits patterns, was observed at K = 8. Common bean landraces and cultivars from the *Albus* group clustered mainly in Mesoamerican M (red in Fig. 2c) (84%) and Andean Ab (dark blue) (80%) subgroups. Genotypes of *Griseus* form predominated in Andean Ae (light blue) (73.9%) and Aa (green) (41.2%) subgroups. *Versicolor* (47.1%) and *Roseus* (42.1%) bean accessions were most numerous in Ac (yellow) subgroup. The most diverse was subgroup Ad (pink), comprising all seed forms. The smallest number of accessions were classified in subgroups Af (orange) (three accessions belonging to *other* forms) and Ag (brown) (two accessions from *Versicolo*r and one from *other* forms).

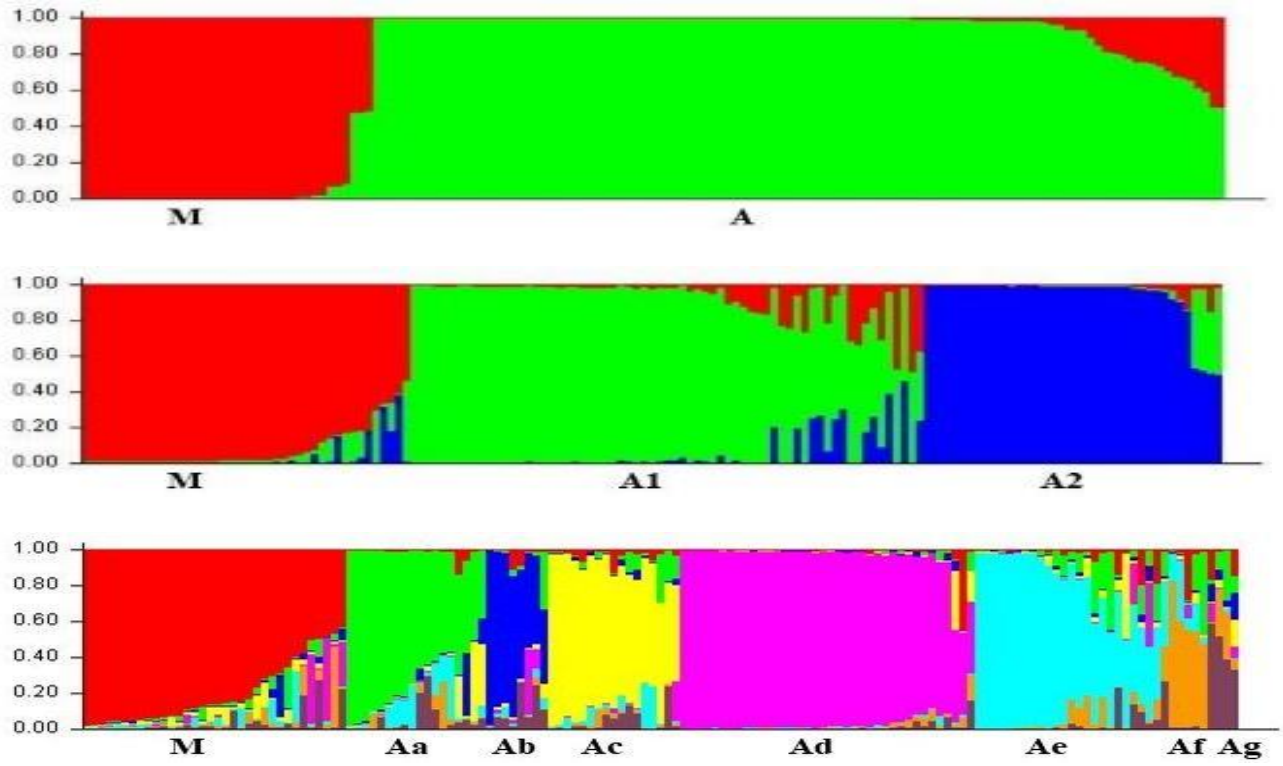

Figure 2. Estimation of population structure for common bean germplasm for a) K=2, b) K=3 and c) K=8 using STRUCTURE software; group generated within Mesoamerican gene pool (M); groups generated within Andean gene pool (A, A1, Aa, Ab, Ac, Ad, Ae, Af, Ag)

The relationships among the genotypes were assessed in more detail by hierarchical cluster analysis (Figure 3). The Neighbour joining-based dendrogram divided 136 genotypes and reference

accessions into two main clusters (gene pools), Mesoamerican and Andean, with additional subclusters identified within each main cluster.

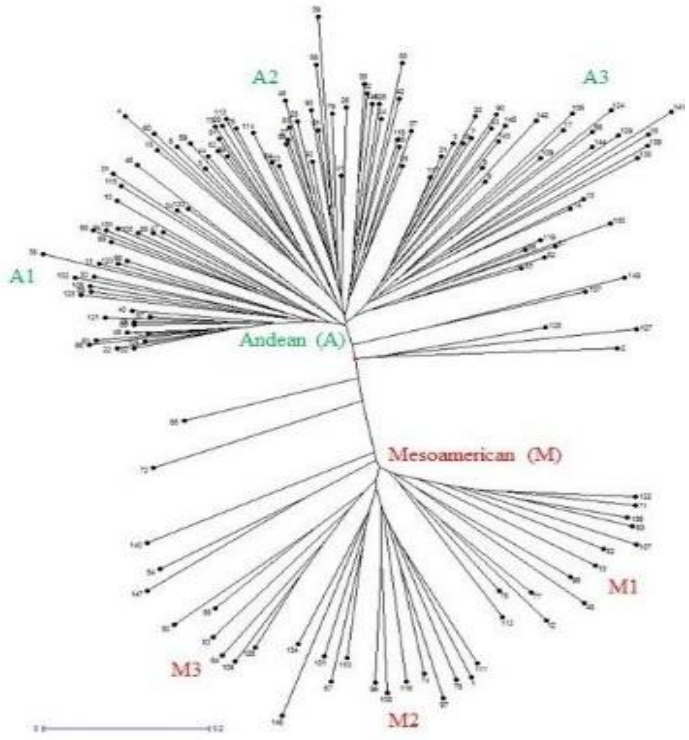

Figure 3. Neighbour joining tree of 136 landraces and cultivars and 13 reference accessions based on similarity matrix (simple matching coefficient)

In total, 32 genotypes (23.5%) were classified in Mesoamerican (M) cluster. Three subclusters are recorded within cluster M. Subcluster M1 included 9 landraces and 3 cultivars (Pasuljica P1, Panonski gradištanac, Belko) with seed weights from small to large. Subcluster M2 consisted of 12 landraces and 6 cultivars (Balkan, Biser, Panonski tetovac, Poboljšani gradištanac, Galeb, Maksa) with medium to large seeds. Two landraces and one cultivar (Medijana) clustered in subcluster M3. All accessions in this group had medium seed weight. Great majority of accessions in cluster M belonged to the *Albus* form.

The Andean (A) cluster comprised 75% of studied landraces and cultivars in total. Subluster A1 consisted of 31 landraces and 2 cultivars (Sremac and Slavonski žutozeleni). Accessions largely belonged to *Grisues*, followed by *Versiclor* in form. In total, 79% of genotypes in subcluster A1 corresponded to subgroup A2 identified in STRUCTURE analysis when K=3, while the rest were of

admixed origin. Therefore, it can be suggested that genotypes comprising these two subclusters represent novel variation, which was created in this region or was introduced from different sources.

Andean subcluster A2 in NJ dendrogram consisted of 40 landraces and 2 cultivars (Oplenac, Zlatko). Landraces and cultivars predominantly belonged to *Grisues* form (48%); however, other seed forms were observed in smaller number – *Roseus*, *Versicolor* and *Aureus*. Only two landraces had a white seed coat. Subcluster A3 comprised 22 landraces, three cultivars (Rozalija, Žutotrban, Aster) and seven reference accessions. The great majority of accessions belonged to *Versicolor* (32%) and *Albus* (28%) forms. Five landraces (L2, L54, L59, L109 and L110) and two reference accessions (PHA390 and PHA131) deviated from this grouping pattern and were separated on the dendrogram.

4. Discussion

For proper conservation, assessment of breeding value and organization and structuring of breeding programs, it is essential to identify levels of phenotypic and genetic diversity preserved in the germplasm of any crop species (Mhlaba et al., 2018). Common bean accessions examined in this paper have already been characterized for their phenotypic variability, while origin and dissemination of local bean germplasm from Serbia was assessed based on variation of phaseolin types (see Savić et al., 2020). Therefore, in order to further reveal genetic diversity preserved in studied germplasm, relatedness of landraces and cultivars on a molecular level, as well as their structure within and beyond gene pools of origin, the present study was performed, which should serve as complementary to the previous one.

Results of this research indicated that substantial allelic diversity was preserved in the germplasm from Serbia and reference accessions. Total number of alleles (Na) and gene diversity (He) were notably higher when compared to germplasm from Portugal, Italy and Croatia (Leitao et al., 2017; Carović-Stanko et al., 2017; Gioia et al., 2019). On the other hand, average number of alleles per locus was comparable to those reported in common bean core collection by Blair et al. (2009). Even though our material was collected from a relatively narrow geographic region and was smaller in size compared to the other research, obtained result could be due to high percentage of rare alleles (68.1%) identified in this paper. In addition, this research material included samples, mainly landraces, from both gene

pools belonging to various seed forms that represented great morphological diversity, which could have affected allelic variability recorded in present study. In addition, the geographic position of Serbia, which is a familiar trade and migration crossroad from east to west, might have been significant in shaping common bean diversity found in this region (Vasić et al., 2009).

Genetic variability was also measured as the amount of actual or potential heterozygosity. Observed heterozygosity (Ho) represents the level of heterozygous individuals in the population compared with expected heterozygosity (He) which reflects the genetic diversity at the specific loci along genotypes due to the degree of its out-crossing potential (Štajner, 2010; Pipan et al., 2013). In the case when Ho is equal to He, it means that the population is in Hardy-Weinberg equilibrium, among other random crosses. In addition, the small deviations between average Ho and He (0.01, Table 2) can indicate uniform abundance of alleles along Serbian common bean germplasm that could reflect their common genetic origin. Moreover, there are some loci where the deviation between Ho and He is higher than 0.3, i.e. loci ATA004, ATA007, ATA010, BM210 and ATA145 (Table 2). Those loci could be highly applicable to evaluate cross-pollination potential of common bean under filed conditions.

Gene diversity (He) recorded in this study, which is not dependable on the sample size, was much higher compared to the results of Leitao et al. (2017) and Carović-Stanko et al. (2017). On the other hand, it mostly corresponded to that found in bean germplasm from Western Balkan countries (former Yugoslav republics) by Maras et al. (2015). This could be due to frequent material exchange, gene flow between the countries that constituted former Yugoslavia, and a similar set of markers used. It was also revealed that gene diversity (He) of each bean group generated according to seed form in this work was larger compared to that observed for common bean market classes from USA by Gioia et al. (2019). Bearing in mind that mentioned authors investigated elite advanced cultivars compared to landraces analysed in this research, it could be suggested that breeding interventions have narrowed the genetic basis of elite material in comparison to landraces. This was also proven with more allelic variants observed among landraces (405) compared to cultivars (233) in our study. Another interesting fact is that a larger percentage of rare alleles were found in landraces (67.6%) in relation to cultivars (29.6%), even though other parameters of genetic diversity were quite similar for these two groups, which also corresponded to values found for the entire collection. This revelation is important from a

breeding perspective, allowing breeders to use this unexplored variability preserved among landraces and cultivars in their advantage. Conversely, allelic variability differed among the groups. Accessions from *Albus* and *Griseus* forms proved to be the most variable, which could also be related to a proportionally larger number of accessions in these seed forms. Therefore, differences in allelic variability found in various other research could be in line with nature, number and variability of accessions assessed, geographic origin of studied material, DNA isolation and detection methods.

The genetic structure of the studied germplasm, on primary level, corresponded to familiar differentiation of common bean accessions according to gene pool affiliation, Mesoamerican or Andean. These results are in accordance with various investigations of *Phaseolus vulgaris* worldwide (Sicard et al., 2005; Kwak and Gepts, 2009; Blair et al., 2010; Raggi et al., 2013; Bitocchi et al., 2017). Separation of Serbian accessions into two recognized gene pools have already been shown based on phaseolin and phenotypic variation, with a considerably larger proportion of accessions belonging to the Andean gene pool (Savić et al., 2020), which is in accordance with results presented in this paper. The prevalence of Andean in contrast to Mesoamerican accessions, along with congruency in bean accessions clustering according to gene pool of origin (estimated at 95%) using different methods in these two research, was determined. It was also shown that a chosen set of SSR markers, including the combination of the P*v*SHP1 markers, proved their usefulness and efficiency in discriminating bean accessions according to the gene pool affiliation, as suggested by Nanni et al. (2011), Maras et al. (2015, 2016), Pipan and Meglič (2019).

Moreover, application of molecular versus phenotypic markers revealed further subdivision of the studied germplasm from Serbia. Additional variation in respect to two recognized gene pools was identified in both STRUCTURE analysis when K=3 (subgroup A2) and based on genetic distance analysis combined with NJ dendrogram (subcluster A1). Accessions with membership coefficients of these two groups largely corresponded to each other. It was also acknowledged that this additional variation is concentrated within the Andean gene pool, which is in line with findings of Maras et al. (2015) for bean germplasm from Western Balkan countries, Raggi et al. (2013) for Italian and Leitao et al. (2017) for Portuguese beans. It is believed that potential sources of this distinctive variation could be accessions with admixed genetic bases derived from inter-gene pool crosses. Apart from that, it is

probable that unique variation was generated in this geographic area as a result of genotypic and phenotypic adaptation to local growing conditions during long period of cultivation. For the Serbian common bean germplasm, it was observed that mainly landraces with greenish-yellow (*Griseus*) and mottled (*Versicolor*) seeds comprised the mentioned clusters, and were marked as new variation. Since these seed types, apart from white-seeded cultivars and landraces, are favourite among the bean producers, it is possible that new variation is a result of farmer's selection towards most tolerant and high yielding landraces in those seed forms.

The theoretical speculation mentioned above is supported by the revelation of putative hybrid genotypes in STRUCTURE analysis. In the case where phaesolin type T predominated among these accessions, it was determined that genetic bases of this material was also within the Andean gene pool. Observed frequency of putative hybrids in this study was quite low (8.1%), but nonetheless in accordance with results of Blair et al. (2010), Gioia et al. (2013) and Maras et al. (2015). On the contrary, Angioi et al. (2010) observed larger percentage (44%) of accessions derived from inter-gene pool hybridization indicating a high contribution of admixed genotypes in the European common bean. Santalla et al. (2002), Logozzo et al. (2007) and Gioia et al. (2013) highlighted the significance of identification of such accessions in certain bean germplasm collections and their breeding value. Putative hybrids might possess new and interesting combination of traits created in inter-gene pool crosses, which could be related to higher adaptability to environmental stress, tolerance to pests and pathogens, better productivity and overcome negative correlation between seed weight and yield potential.

Relatedness of landraces and cultivars was discussed based on genetic distance analysis associated with Neighbour-joining clustering method, while subsequent genetic structure was revealed when K=8 was considered in STRUCTURE analysis. In both methods, it was obvious that landraces and cultivars formed subgroups according to their phenotype (with several exceptions). Arguably, however, a genetic distance-based method might better assist in differentiation among accessions of similar phenotypes and in selection of more genetically distant parenting components for breeding. In the case of common bean, it would support breeding of cultivars in specific seed type (form) with improved productivity. Different types of clustering methods in combinations with genetic distance

calculations were applied. Observing the fact that both landraces and cultivars were used within calculations, the combination of NJ and simple matching coefficient was the best choice to evaluate and visualise the genetic relations and distribution of Serbian genotypes according their genetic origin (gene pool determination). Moreover, the distribution of genotypes among clusters (A1-A3; M1-M3) on Figure 3 is also correlated with seed characteristics for both, Andean and Mesoamerican group, respectively.

A larger number of groups distinguished among landraces indicate existent diversity of this material, despite the fact that farmers have maintained landraces during longer period on the farms close to each other, exchanged seeds with surrounding farms and among themselves. Our results are in accordance with research of Masi et al. (2009) and Raggi et al. (2013) who made similar observations. Even though landraces clustered largely according to the seed traits, some deviations of this pattern were observed. The largest discrepancy was detected among accessions belonging to the *Albus* seed form. Even though these accessions are mainly of Mesoamerican origin, large white-seeded types were also observed in Andean groups. There are two possible explanations of this finding. Firstly, breeding intervention in Serbia were mostly done within white-seeded beans, which could have resulted in hybridization between the gene pools. Secondly, common bean landraces with white large seeds, belonging to Nueva Granada race were introduced and are grown in Serbia. In addition, accessions of *Roseus* and *Versicolor* group often clustered together. Although these accessions are usually distinguished by their phenotype, they were not well separated on molecular level in this research.

Distribution of Serbian cultivars in distinctive, genetically diverse subclusters in NJ dendrogram is a result of various selection and breeding criteria over time. It is well known that there is accepted tendency to satisfy market demands in term of making cultivars that phenotypically correspond to specific market class, or seed form (Geravandi et al., 2020). In various timeframes of common bean breeding in Serbia, different available material was used, which also included introduction of foreign germplasm for breeding purposes. This, together with adaptation of newly created material to environmental and growing conditions at the time, resulted in genetic divergence of Serbian common bean assortment. On the other hand, there were cases when two or more cultivars were

more closely positioned on the dendrogram. The most probable cause of this phenomenon is the common genetic origin they share, belonging to the same ancestral line.

In a conclusion, examined landraces and cultivars displayed marked genotypic variation, which allowed detailed description of diversity present in common bean germplasm from Serbia, accompanying the information on phenotypic variability previously assessed. Classification and structuring of the accessions in accordance within and beyond gene pool of origin should facilitate conservation strategies and breeding of this material. A combination of phenotypic and molecular data should allow researchers to make a selection of smaller number of accession for the core collections, which will further be assessed for their breeding value (productivity, nutritional value, tolerance to biotic and abiotic stress). As a result of the information presented in this research, it is hoped that the study of common bean genetic diversity in the Western Balkans and the rest of Europe has been purposefully expanded.

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