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Research article

Estimation of genetic diversity and relationship in sugar beet pollinators based on SSR markers

Ksenija Taški-Ajduković *, Nevena Nagl, Živko Ćurčić, Miroslav Zorić

Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia

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ABSTRACT

Background: Genetic diversity studies are important for the selection of parents with a greater combination capacity that, when crossed, increase the chances of obtaining superior genotypes. Thus, 26 polymorphic simple sequence repeat (SSR) primers were used to assess the genetic diversity of 140 individual samples from 12 diploid sugar beet pollinators (pollen parents) and two cytoplasmic male sterile (cms) lines (seed parents). Eight pollinators originated from three research centers in the United States Department of Agriculture, while four pollinators and cms lines were from the Institute of Field and Vegetable Crops, Novi Sad, Serbia.
Results: In total, 129 alleles were obtained, with a mean of 3.2 alleles per SSR marker. The observed heterozygosity ranged from 0.00 to 0.87 (mean = 0.30). Expected heterozygosity and Shannon's information index were the lowest for marker BQ590934 and the highest for markers SB15s and FDSB502s; the same markers were the most informative, with PIC values of 0.70 and 0.69, respectively. Three private alleles were found in pollinator EL0204; two in pollinator C51; and one in pollinators NS1, FC221, and C93035. Molecular variance showed that 77.34% of the total genetic variation was attributed to intrapopulation variability. Cluster and correspondence analysis grouped sugar beet pollinators according to the breeding centers, with few exceptions, which indicate that certain amount of germplasm was shared, although centers had their own breeding programs.
Conclusions: The results indicate that this approach can improve the selection of pollinators as suitable parental components and could further be applied in sugar beet breeding programs.

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1. Introduction

Sugar beet (*Beta vulgaris* L.) is an economically important crop in moderate climate zones and accounts for approximately 25% of the worldwide sugar production [1]. In addition to producing sugar, the sugar industry annually produces large amounts of by-products such as molasses and sugar beet pulp (SBP), which are widely used as feed supplements for livestock [2,3]. Owing to its high content of readily fermentable sugars, SBP and molasses hold a great potential for energy-efficient bioethanol production [4,5]. They also present an interesting feedstock for the alcohol, yeast, and pharmaceutical industries.

Sugar beet is a cross-pollinated species with breeding based on crosses between diploid cytoplasmic male sterile (cms) lines (seed parents) and mainly diploid pollinators (pollen parents). The genetic base of the commercial sugar beet varieties has been narrow for some time, mainly because of the repeated use of a limited number of genotypes as parents in breeding programs [6]. This is likely to cause inbreeding depression and reduced genetic variability, which in turn can lead to genetic plateaus in sugar beet [7]. Characterization of germplasm is a matter of prime concern to reduce the effort and expense spent on the identification of suitable parent lines and boost genetic improvement. Therefore, a better understanding of genetic variability within and between populations used as progenitors, as well as their relationship, is important for the efficient selection of hybrid crosses and further improvements of the breeding programs [8]. For that purpose, techniques based on molecular markers analysis have been recognized as the most useful [9,10].

Different types of DNA-based markers have been used for genetic analyses in sugar beet, including restriction fragment length polymorphism (RFLP) [11,12,13], amplified fragment length polymorphisms (AFLP) [14,15], randomly amplified polymorphic DNAs [12,16,17], intersimple sequence repeat (ISSR) [18], single

* Corresponding author.
E-mail address: ksenija.ajdukovic@ifvcns.ns.ac.rs (K. Taški-Ajduković).
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Table 1
Origin of sugar beet pollinators.

Pollinators	Origin
EL0204	USDA-ARS, Michigan, USA
EL53	USDA-ARS, Michigan, USA
NS1	IFVCNS, Serbia
NS2	IFVCNS, Serbia
NS3	IFVCNS, Serbia
NS4	IFVCNS, Serbia
CR10	USDA-ARS, Salinas, USA
C930-35	USDA-ARS, Salinas, USA
CZ25-9	USDA-ARS, Salinas, USA
C51BM	USDA-ARS, Salinas, USA
FC221	USDA-ARS, Fort Collins, USA
FC220	USDA-ARS, Fort Collins, USA

IFVCNS — Institute of Field and Vegetable Crops in Novi Sad, Serbia; USDA-ARS — research centers of the United States Department of Agriculture, Agricultural Research Service.

nucleotide polymorphisms [19,20], and simple sequence repeats (SSRs) [15,21,22].

Among the variety of molecular markers, SSR markers have found widespread application because of their high reproducibility, hypervariability, multiallelism, codominant inheritance, extensive genome coverage, chromosome-specific location [23,24], and easy automated detection by polymerase chain reaction (PCR) [25]. In sugar beet, a few hundred SSR markers have been developed and used for the development of genetic maps [15,21], investigation of gene flow [26,27], and population genetic analyses [28,29,30]. However, there are few reports on the genetic diversity of elite sugar beet germplasm using SSRs [31,32,33].

The aim of this study was to assess the genetic diversity of sugar beet pollinators from different breeding centers and estimate their genetic relationship through SSR markers, with the final aim to select pollinators as a potential parental population to develop new breeding material with a broadened genetic base.

Table 2
SSR primers used for genetic diversity assessment.

Primer name	Primer sequence	Linkage group	Reference
521.6	5′-AATAAAAAAATGTTAAAAAAGCAC-3′ 5′-AAAACAGAGGTAAATCGGTCAAAC-3′	LG1	McGrath et al. [15]
BQ583448	5′-TATTGTTCTAAGGCACGCA-3′ 5′-CGCTATCCTCTTCGTCAA-3′	LG1	McGrath et al. [15]
BI096078	5′-CAATTCCCCTTCCAAAAACA-3′ 5′-GCTAAACCAAACCCATGTGC-3′	LG1	McGrath, personal communication
FDSB1300	5′-AATTTAAACGCGAGAGCAGC-3′ 5′-TCAGCTTCTGGGCTTTTTGT-3′	LG2	Laurent et al. [21]
EG551958	5′-ATAACTCTCGCCTACAAATGA-3′ 5′-TCTACCTTGCCCGTAAACT-3′	LG2	McGrath personal communication
BI543628	5′-GAACTCCTTTGACAGCATCTT-3′ 5′-CCTTCAGCATCTCTCTCTC-3′	LG3	McGrath et al. [15]
SB06	5′-AAATTTTCGCCACCACTGTC-3′ 5′-ACCAAAGATCGAGCGAAGAA-3′	LG4	Richards et al. [28]
SB07	5′-TGTGGATGCGCTTTCTTTTC-3′ 5′-ACTCCACCCATCCACATCAT-3′	LG4	Richards et al. [28]
DX580514	5′-CCTAATGCCTCTTGTGCTAA-3′ 5′-ATAGACCTCCTTGTGGGAAAC-3′	LG4	McGrath, personal communication
BU089565	5′-GCTTGGGGCACTTGGCATTC-3′ 5′-CTATACGTTGTGACCACGTG-3′	LG5	McGrath et al. [15]
EG552348	5′-GGTGGTTATGCTCCTCCT-3′ 5′-GGCTTTAGTCTTATTGCTGTG-3′	LG5	McGrath, personal communication
SB15	5′-CACCCAGCCTATCTCTCGAC-3′ 5′-GTGGTGGGCAGTTTTAGGAA-3′	LG5	Richards et al. [28]
SB04	5′-ACCGATCACCAATTCACCAT-3′ 5′-GTTTTGTTTTGGGCGAAATG-3′	LG5	Richards et al. [28]
BvGTT1	5′-CAAAAGCTCCCTAGGCTT-3′ 5′-ACTAGCTCGCAGAGTAATCG-3′	LG6	Viard et al. [35]
FDSB568	5′-TTCTGGGGATGATTTCTTCG-3′ 5′-CCGGGACAGAGAGAACAGAG-3′	LG6	Laurent et al. [21]
BQ487642	5′-ATCAAACTCCTCCTCTGTCTC-3′ 5′-TTACAACAACAACAACAACAAA-3′	LG6	McGrath et al. [15]
FDSB1011	5′-CAACTTATTTAAGCCTTTTAGTGC-3′ 5′-GATCCATTTATTTCGTGTTGA-3′	LG7	McGrath et al. [15]
FDSB502	5′-GCAAAAACCCAAAACCCTTT-3′ 5′-TTTCTCTCTCCTCCTCTTCCTC-3′	LG7	Laurent et al. [21]
FDSB990	5′-TCTCACCTGAAATCCGAACC-3′ 5′-CCATCCGTAACTCGGTGACT-3′	LG7	Laurent et al. [21]
FDSB1250	5′-TTCACCGCCTGAATCTTTTC-3′ 5′-CGACGAAGAATCGGGTAAAA-3′	LG7	Laurent et al. [21]
BI073246	5′-ACGAGGAACAAATCCACACC-3′ 5′-CAACACCAGGTCGATGTTTG-3′	LG8	McGrath, personal communication
BQ582799	5′-CCTTGCCCGCTCTTTTTCA-3′ 5′-CTCCCGTAGGCGTCTCTTCAT-3′	LG8	McGrath, personal communication
BQ590934	5′-ATCTCTGCCTCTACCGCC-3′ 5′-GCATTTGTATTGTTATCTCTCTC-3′	LG8	McGrath, personal communication
FDSB1007	5′-ATTAGAATAGCATCAATTGTGG-3′ 5′-CCTTATAGTTGGAATTGAGAAA-3′	LG8	McGrath et al. [15]
FDSB1001	5′-ACTTCAACCACTATCACAAAGTGAG-3′ 5′-ATCTTATGCTGCCATGACCA-3′	LG9	McGrath et al. [15]
FDSB1033	5′-GCTGAGATGATGTTTGTTAGGGC-3′ 5′-TTCAAATCGCCATCTCCCAG-3′	LG9	McGrath et al. [15]

Table 3
PCR programs used for SSR primers amplification.

Tm (°C)	SSR primers
52	521.6
53	BQ583448, BQ487642
54	FDSB1011, FDSB502, BvGTTT1, BIO96078, SB04
55	**EG551958,** FDSB1007
56	SB06**, EG552348,** SB07, FDSB1300, DX580514, FDSB1250, FDSB1001, FDSB568
57	BI543628, BQ582799
58	BUO89565, SB15, **FDSB1033,** FDSB990, BQ590934, BIO73246

Regular — amplification is the same with both types of programs; Underlined — better amplification with regular program; **Bold** — better amplification with touchdown program; Tm — melting temperature.

2. Material and methods

2.1. Material

A total of 140 individual samples were collected from 12 multigerm sugar beet pollinators (pollen parents) and two cms lines (seed parents). Four pollinators were developed at the Sugar Beet Department of the Institute of Field and Vegetable Crops in Novi Sad, Serbia (IFVCNS), and eight came from research centers in the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) (Table 1). Two cms lines (cms1 and cms2) originating from IFVCNS were also used.

2.2. DNA isolation and PCR

The DNA of 140 individual samples was isolated according to the protocol given by Somma [34]. For diversity studies, 26 polymorphic SSRs were selected [15,21,28,35], depending on the number and size of the amplified fragments in the preliminary investigation (data not shown) and placement in a certain linkage group (Table 2). Considering the primers melting temperature (Tm), a series of regular and touchdown amplification programs were developed, with annealing temperatures ranging from 52 to 58°C (Table 3).

PCR conditions for regular SSR amplification were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at an optimized temperature for 40 s, and elongation at 72°C for 50 s. The final elongation was performed at 72°C for 5 min. The "Touchdown" PCR program included initial denaturation at 94°C for 2 min, followed by cycles of 94°C for 30 s, annealing at an optimized temperature for 30 s, and 72°C for 50 s. The annealing temperature was decreased by 0.8°C per cycle in subsequent cycles until 47°C was reached. Amplification products were subsequently amplified for 30 cycles at 94°C for 30 s, 47°C for 30 s, and 72°C for 50 s, and the final extension step was at 72°C for 5 min. PCR was performed in a 25-μL reaction volume containing 2.5 μL buffer, 0.2 mM of each dNTP (Thermo Scientific), 0.4 μM of primers (Metabion), 1.5 U DreamTaq DNA polymerase (Thermo Scientific), and approximately 50 ng DNA. Tpersonal thermocycler (Biometra) was used for DNA amplification.

Table 4
Genetic diversity statistics of SSR markers in sugar beet.

SSR marker	Na	Ne	Ho	He	I	PIC
521.6	4	2.84	0.51	0.65	1.19	0.59
BIO96078	3	2.03	0.17	0.51	0.82	0.43
BQ583448A	3	2.58	0.30	0.61	1.01	0.54
BQ583448B	3	2.93	0.33	0.66	1.09	0.59
FDSB1300S	2	1.89	**0.00**	0.47	0.66	0.36
FDSB1300F	3	1.95	0.20	0.49	0.71	0.36
EG551958	3	1.99	0.14	0.50	0.74	0.39
BI543628A	2	1.70	0.54	0.41	0.60	0.33
BI543628B	2	1.97	**0.87**	0.49	0.69	0.37
BI543628C	3	2.06	0.40	0.51	0.76	0.39
SB07s	4	2.63	0.06	0.62	1.13	0.57
SB07f	2	1.77	0.02	0.43	0.63	0.34
SB06	4	3.21	0.16	0.69	1.28	0.64
DX580514	5	1.91	0.32	0.47	0.93	0.44
SB04	4	3.43	0.34	0.71	1.30	0.66
SB15s	5	**3.94**	0.48	**0.75**	**1.42**	**0.70**
SB15f	3	2.56	0.11	0.61	1.00	0.56
EG552348	3	2.09	0.35	0.52	0.80	0.41
BU089565A	2	1.86	0.46	0.46	0.66	0.36
BU089565B	4	2.69	0.28	0.63	1.09	0.55
FDSB568s	4	3.23	0.49	0.69	1.24	0.63
FDSB568f	3	2.09	0.09	0.52	0.88	0.46
BVGTT1	2	1.49	0.36	0.33	0.51	0.27
BQ487642A	3	2.20	0.50	0.55	0.86	0.44
BQ487642B	3	2.25	0.49	0.55	0.89	0.46
BQ487642C	3	2.16	0.58	0.54	0.84	0.43
FDSB990s	4	2.92	0.35	0.66	1.13	0.59
FDSB990f	3	2.65	0.02	0.62	1.03	0.55
FDSB502s	5	**3.72**	0.04	**0.73**	**1.43**	**0.69**
FDSB502f	3	2.23	0.39	0.55	0.87	0.45
FDSB1250	3	2.28	0.19	0.56	0.92	0.48
FDSB1011A	4	2.74	0.53	0.63	1.07	0.56
FDSB1011B	3	1.68	0.16	0.40	0.73	0.37
FDSB1007	2	1.42	0.11	0.30	0.47	0.25
BQ590934	3	**1.33**	0.26	**0.25**	**0.44**	**0.22**
BQ582799	3	2.20	0.37	0.55	0.86	0.44
BIO73246	3	2.01	0.32	0.50	0.71	0.37
FDSB1033s	4	1.98	0.12	0.49	0.93	0.45
FDSB1033f	5	2.56	0.46	0.61	1.10	0.53
FDSB1001	2	1.99	0.28	0.50	0.69	0.38
Average	3.22	2.33	0.30	0.54	0.90	0.46

Na — observed number of alleles; Ne — effective number of alleles; Ho — observed heterozygosity; He — expected heterozygosity; I — Shannon's information index.
bold - the lowest values, bold, underlined - the highest values.

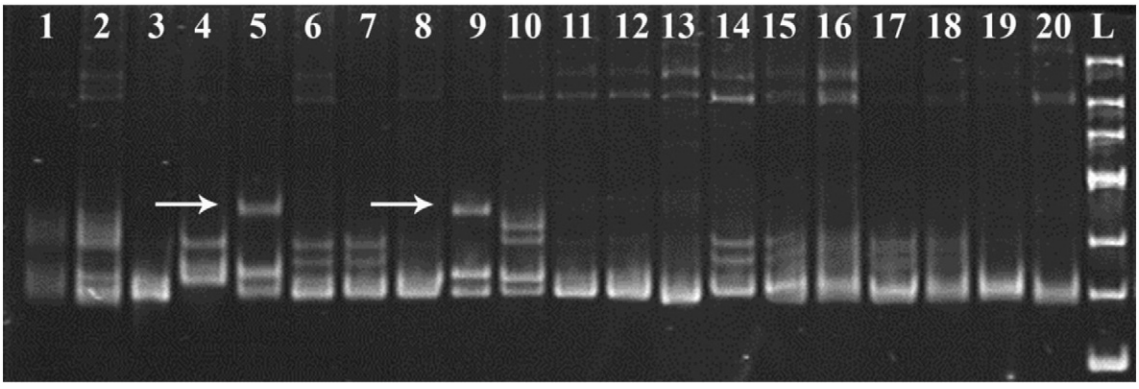

Fig. 1. Products of PCR with primer SB15 showing the occurrence of the private allele on sugar beet pollinator lines. 1–10: samples of pollinator EL0204, 11–20: samples of pollinator EL53, L – 1 kb – GeneRulerTM 100 bp ladder Plus.

Amplification products of primer BIO96078 were electrophoretically separated on 2% agarose. Product of primers BQ583448, FDSB1300, BI543628, SB07, DX580514, EG552348, BvGTT1, FDSB568, BQ487642, FDSB502, FDSB990, FDSB1007, FSSB1001, and FDSB1033 and product of primers SB04 and 521.6 were separated on 2.5% and 3% MetaPhor agarose gels containing 0.005% ethidium bromide, respectively. The remaining PCR products were separated on 4% (primer BQ582799), 5% (primer EG551958), 6% (primers BU089565, FDSB1011, and BI073246), and 8% (SB06, SB15, FDSB1250, and BQ590934) polyacrylamide denaturation gels; detected by ethidium bromide staining; and visualized under UV light. The band size was estimated by comparison with GeneRuler 50 bp DNA Ladder (Thermo Scientific).

2.3. Data analysis

The SSR profiles were scored according to the size (bp) of the amplified fragments of all individual samples from the tested populations on the basis of the visual inspection of electrophoretic patterns. The polymorphism information content (PIC) [36], observed and effective number of alleles [37], observed and expected heterozygosity [38], and Shanon's information index [39] were calculated for all SSR loci using PowerMarker 3.25 [40] and POPGENE 1.32 [41] software packages. Variance components among and within the tested populations were estimated by analysis of molecular variance (AMOVA) [42], using the ARLEQUIN 3.11 software [43].

Genetic distance between each pair of population was calculated using the equation given by Nei [44]. On the basis of the resulting matrix, a dendrogram was constructed using the neighbor-joining algorithm and generated by TREE display option as available in NTSYSpc software package version 2.11a [45]. Spatial representation among the tested individuals and populations was examined by correspondence analysis implemented in the R software [46].

3. Results and discussion

In all 140 samples, amplification with tested SSR primers resulted in PCR products that varied in size and number. In most cases, the quantity and number of amplification products was the same in regular and touchdown versions of the PCR program (Table 3). Amplification with primers SB04, FDSB1300, and FDSB1001 was better with regular program, whereas primers EG551958, EG552348, BQ591109, and FDSB1033 amplified better with the touchdown version.

Primers BQ583448, FDSB1300, SB07, SB15 (Fig. 1), BU89565, FDSB568, FDSB990, FDSB502, FDSB1011, and SB1033 had multiple products from two independent loci, whereas primers BI543628 and BQ487642 gave products from three loci. In total, 40 SSR loci had 129 different alleles. The number of alleles varied from two to five, with an average of 3.22 per locus (Table 4), which is in agreement with the results of Fugate et al. [47] but is lower than those in Desplanque et al. [13], Richards et al. [28] and Viard et al. [35] who worked on wild forms of *Beta vulgaris*. In the study by Smulders et al. [22], the average number of alleles was also higher, which is expected, because the study included more diverse plant material. Alleles found only in a single population were classified as private. Eight private alleles were detected across the tested pollinators. Pollinator EL0204 had three private alleles (Fig. 1); pollinator C51 had two private alleles, while pollinators NS1, FC221, and C93035 had one private allele. Among the analyzed SSR loci, seven revealed private alleles, suggesting that these markers could be useful in cultivar identification and registration. The importance of private alleles in genetic diversity and genetic distinctiveness and as a guide for choosing parents in a breeding program has been thoroughly been discussed by Chen et al. [48].

The observed heterozygosity for SSR markers ranged from 0.00 to 0.87, with a mean value of 0.30, whereas the expected heterozygosity varied from 0.25 to 0.75, with an average of 0.54 (Table 4). The expected heterozygosity and Shannon's information index were the

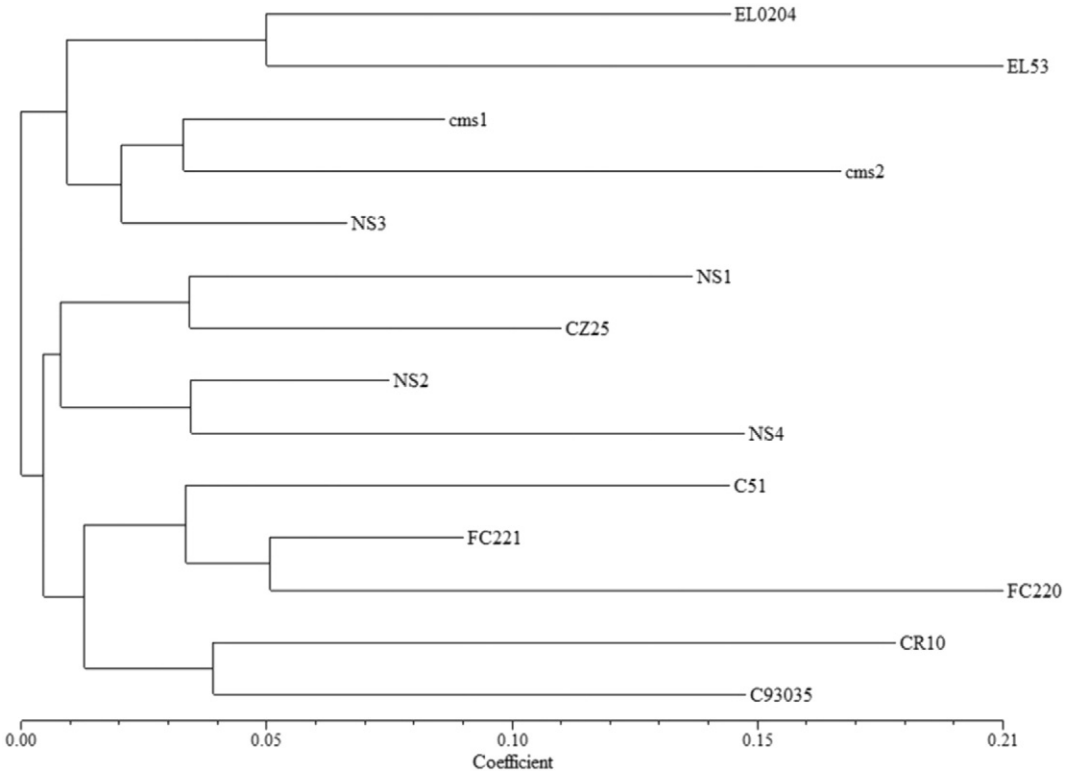

Fig. 2. Neighbor-joining cluster analysis of sugar beet based on Nei's genetic distances.

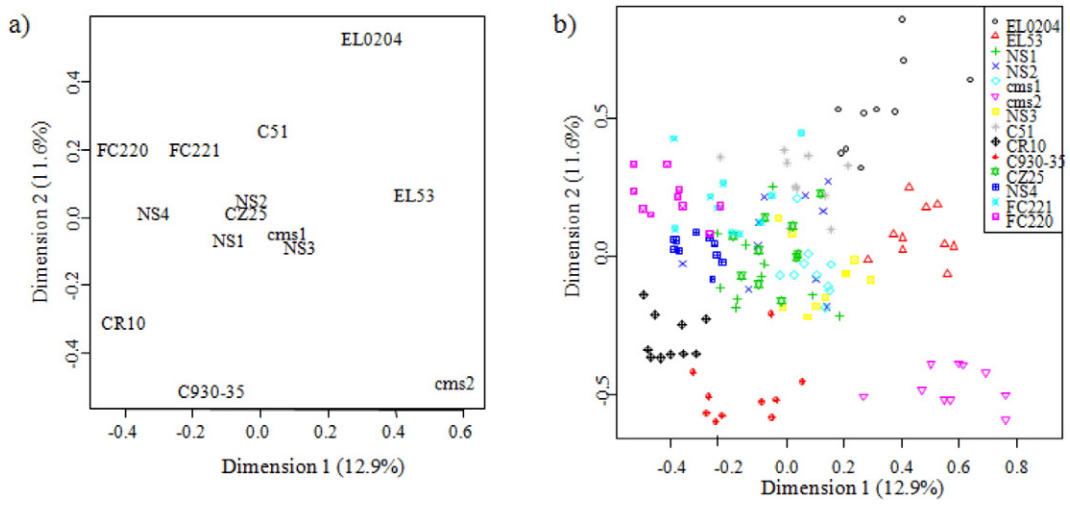

Fig. 3. Association among the tested sugar beet populations (a) and individuals (b) revealed by correspondence analysis.

lowest for marker BQ590934 (0.25 and 0.44, respectively) and the highest for markers SB15s (0.75 and 1.42, respectively) and FDSB502s (0.73 and 1.43, respectively). The latter two markers were the most informative, with PIC values of 0.70 and 0.69, respectively. High PIC values recorded in the study (PIC = 0.22–0.70, mean = 0.46) indicated both the highly informative nature of the SSR markers and the diversity of the used populations. The detected heterozygosity was lower than that in McGrath et al. [6], Nagl et al. [17], and Poulsen et al. [49], which was not surprising because different types of markers were used in the mentioned studies. Nevertheless, the estimated heterozygosity was similar to Smulders et al. [22] and Li et al. [31], where SSR markers also were used to estimate the genetic diversity in sugar beet germplasm.

The AMOVA indicated that most (77.34%) of the molecular variations in the tested sugar beet populations existed within populations, and only 22.66% was among populations. This is in agreement with similar studies on cultivated sugar beet by Abbasi et al. [33] and De Riek et al. [50]. The gene diversity within individual plants increases in the breeding system where separate gene pools are used for the development of paternal and maternal parents [35]. Utilization of parental pools containing a large amount of genetic diversity may contribute to the fact that majority of the genetic variation of the crop is present within hybrid varieties [50].

The neighbor-joining dendrogram, constructed on the basis of genetic distance matrix, showed that sugar beet populations were grouped into two major clusters (Fig. 2). The first cluster was divided to sub-cluster containing pollinators EL0204 and EL53, from USDA-East Lansing and sub-cluster containing the cms lines cms1 and cms2 and pollinator NS3 from IFVCNS. The second cluster consisted of two sub-clusters. The first sub-cluster grouped the pollinators from IFVCNS breeding program and pollinator CZ25 from USDA-Salinas, which shares a common ancestor with a pollinator from the European breeding program [51]. The second sub-cluster grouped pollinators CR10 and C930-35 from USDA-Salinas and FC220, FC221 (USDA-Fort Collins), and C51 on the other branch. The distinct grouping of C51 compared to other pollinators from USDA-Salinas was probably because half of its germplasm originated from *Beta vulgaris* subsp. *maritima*. Moreover, both pollinators from USDA-Fort Collins descended from C51 [52,53].

Spatial representation of the relative genetic distances among the individuals was provided by correspondence analysis, which also determined the consistency of differentiation among populations defined by the cluster analysis. The first two dimensions explained 12.9% and 11.6% of the total variation, respectively (Fig. 3). The scattering of populations was similar to the results of the cluster analysis, where pollinators were mostly grouped according to their breeding centers. Pollinators from USDA-East Lansing (EL0204 and EL53), USDA-Fort Collins (FC220 and F221), and USDA-Salinas (CR10 and CR930-35) formed distinctive groups, while CZ25 was grouped with pollinators and cms1 from IFVCNS, where some overlapping occurred. C51 was positioned closer to USDA-Fort Collins pollinators than to USDA-Salinas germplasm, just as in cluster analysis. Unlike the cluster, the correspondence analysis also positioned C51 closer to IFVCNS pollinators, indicating its involvement in the development of the IFVCNS breeding program.

In conclusion, the results indicate that the genetic diversity estimates between and within populations depend not only on the type of markers chosen for the particular study but also on the choice of the collections and the analyzed genotypes [35]. The number of individuals sampled per genotype and the number of loci used for genotyping also affect the accurate genotype assignment [54]. Although it has repeatedly been reported that it is difficult to reliably resolve the relationships between closely related sugar beet germplasms, even when a large number of markers are employed [55], the cluster and correspondence analysis relatively proved the distribution of pollinators according to the breeding centers. Few pollinators were grouped differently, indicating that there was overlapping in certain breeding programs and exchange of breeding germplasm. Furthermore, it was evident that the assessment of genetic diversity of sugar beet pollinators from different breeding centers and estimation of their genetic relationship on the basis of SSR molecular markers may provide more accurate information for sugar beet breeders. In future, USDA pollinators with higher genetic distance from the IFVCNS germplasm will be used in crosses to develop new breeding material, with broadened genetic base, which could increase the frequency of genes for desirable traits.

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Conflict of interest

None.

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