ISSN 2744-1636
Bioengineering Studies *Original Research*
Vol. 3, No. 1, September 2022, pp.31-39
https://doi.org/10.37868/bes.v3i1.id226

Genetic diversity analysis of *Capsicum annuum L.* cultivars from Bosnia and Herzegovina using EST-SSR markers

Jasmin Šutković[1]*, Petar Glamočlija[2], Lutvija Karić[2]
[1] Faculty of Engineering and Natural Sciences, International University of Sarajevo, Bosnia and Herzegovina
[2] Faculty of Agricultural and Food Sciences, University of Sarajevo, Bosnia and Herzegovina

*Corresponding author: jsutkovic@ius.edu.ba

Abstract
With a significant economic value, pepper (*Capsicum annuum L., Solanaceae*) represents a major vegetable crop worldwide. Simple sequence repeats (SSR) markers and expressed sequence tags (EST-SSR) are powerful tools for genetic studies and the breeding of pepper. In this study, the genetic variability among local pepper species was assessed by using 8 EST/SSR markers. The pepper varieties were collected locally, based on old sorts, hybrids, and sorts found in markets, with a total of 14 pepper varieties. Mean expected heterozygosity (He) ranged from s 0.30 to 0.83 in the populations studied, whereas the mean polymorphic information content (PIC) was 0.62. The average polymorphic number of alleles per primer was 2.5 per locus. The genetic relationships among the populations revealed by Neighbor-Joining dendrogram showed a clear clustering to three sub-populations making distinct clusters. As expected, all local varieties make up one cluster, together with the hybrid Istra, concluding that this hybrid evolved from the cross-breeding of local varieties. These results indicate and confirm the regional and physiological differences between the pepper sorts.

Keywords: EST-SSR marker; Genetic characterization; *Capsicum annuum L., Solanaceae*; Polymorphism; Phylogeny

1. Introduction

Capsicum annuum L. is a species of the plant genus Capsicum, which includes a wide variety of cultivars such as bell peppers, chili peppers, and paprika. These cultivars have been an important part of human diets and cultures for thousands of years, with evidence of cultivation dating back to at least 7500 BC [1]. *Capsicum annuum* cultivars vary in their size, shape, and level of heat, with some being sweet and mild and others being spicy and hot. The level of heat in a particular cultivar is determined by the concentration of a compound called capsaicin, which is found in the seeds and veins of the pepper [2]. In addition to being a popular food ingredient, Capsicum annuum cultivars have also been used in traditional medicine for their potential health benefits. For example, capsaicin has been shown to have anti-inflammatory and pain-relieving effects (Zhang et al., 2015), and some studies have suggested that consumption of Capsicum annuum cultivars may be beneficial for weight management and cardiovascular health [3]. *Capsicum annuum* cultivars are native to the Americas and are now grown and consumed worldwide [4]. In addition to being an important source of vitamin C and other nutrients, these cultivars are also an important part of many cuisines and cultural traditions. For example, chili peppers are a staple ingredient in many Mexican and South American dishes, and bell peppers are commonly used in European and North American cuisine [1]. These cultivars are grown and consumed worldwide and are an important part of many cuisines and cultural traditions. Bosnia and Herzegovina (BiH) belong to the top of European biodiversity countries. Due to the political and economic

isolation in the past, many traditional crop varieties remained genetically authentic.

Two seed genetic banks exist currently in BiH, where more than 1100 accessions are conserved [5]. Some of these varieties are still used in agricultural production, and some of these are domestic as autochthonous pepper varieties, found mainly in Čapljina region, located in the south-west region of BiH [6].
The high number of pepper varieties in this region requires the preservation of genetic resources as a mandatory measure for diversity conservation. Indeed, through governmental, regional, local, and agricultural associations, the conservation of local pepper verities is partially carried out, but not efficiently. Therefore, there is a need for efficient species management and reliable conservation, which can be achieved through genetic diversity assessment by using DNA markers.
Molecular markers became an efficient tool for the assessment of genetic diversity of plant species. Among various currently used DNA markers, microsatellites or commonly known as Simple Sequence Repeat (SSR) are mostly used [7]. SSR markers result in highly polymorphic, abundant, and reliable results. Further, compared to other DNA markers, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplifies Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP), the utilization of SSR markers represent a cheap procedure for genetic assessment of plant species, being highly reproducible and abundant DNA marker [8, 9]. Simple sequence repeats are motives that occur randomly in the coding and non-coding part of the genome sequence [10].
Several sets of EST-SSR markers have been previously used to detect genetic diversity in pepper germplasm [11], but no studies with the genotypes of Bosnia and Herzegovina are recorded so far. EST-derived SSRs belong to the transcribed regions of DNA and are expected to be more conserved and have a higher transferability rate across species than genomic SSR markers [12].
In the present study the aim is to genetically characterize the pepper cultivars found in Bosnia and Herzegovina.

2. Materials and Methods

2.1. DNA extraction

In total 425 pepper individuals, from14 *Capsicum annuum L* populations were used in this study (Table 1). All the analyzed populations, except Domestic varieties, are currently commercially released onion varieties, being very specific, with high diversity in the public bred material. Domestic varieties were collected from farmers as fresh, fully grown plants, and stored in -20C until DNA isolation.

Table 1. Onion cultivars used in this study

No	*Name of varieties (Capsicum annuum L)*	*Code*	*Sample origin*	*No. of Samples*
1	Grossum, California wonder yellow	CW	USA	20
2	Rotundum, Rotund žuta	RY	Croatia	35
3	Rotundum, Rotund zelena	RZ	Croatia	35
4	Rotundum, Paradajz paprika	RC	Croatia	35
5	Chili ,Corno Di Toro Roso	CD	Italian	20
6	Kolgum, Slonova surla	SS	Croatia	35
7	Somborka	SO	Serbian	30
8	Šoroškari	SR	Serbian	35
9	ISTRA	IS	Croatia	40
10	Giallo Dasti Roso	GS	Italian	20
11	Domestic Faruk -domestic 1	FP	Domestic,BiH	30
12	Domestic Srečko Perić -domestic2	SP	Domestic,BiH	30
13	Domestic Cevas njive-domestic 3	DC	Domestic,BiH	30
14	Domestic Višići-domestic 4	DV	Domestic,BiH	30

The genomic DNA was isolated from dry seeds and the fresh leaves. Plant material (leaves) for the DNA extraction was collected from randomly selected plants on each analyzed site. After harvesting, the seeds and

leaves were pulverized with liquid nitrogen using mortar and pestle. The DNA was extracted according to the modified CTAB (*Cetyltrimethyl ammonium bromide*) protocol [13].

The DNA quality was analyzed on 1% agarose gel and quantified using a Multiscan GO (Thermo Fisher Scientific, USA) and stored in 100µl aliquots in 1 X TE buffer at –20°C until PCR amplification.

2.2. EST-SSR analysis

All PCR reactions were conducted in total volume of 25 µl, containing 2 mM of $MgCl_2$, 1 x PCR buffer, 0.2 mM of dNTPs, DNA Polymerase from Thermus aquatic (5 units/µL) and 10-50 ng of template DNA.
The PCR protocol included two phases, starting with initial denaturation phase at 95 ˚C for 5 min. The first phase is a touchdown (TD) PCR profile, with 20 cycles, starting with 95˚C denaturation for 45s, where the annealing temperature was reduced by - 0.7 ˚C per cycle for 45s, followed by the extension stage at 72 ˚C for 1 min. The second PCR phase, with 15 cycles in total, included 95˚C denaturation for 45s, the annealing temperature for each primer according Fischer and Bachmann (2000) [14], and extension at 72˚C for 1 min. The PCR protocol was completed with the final extension step at 72 ˚C for 7 min.

The PCR products were separated on 3% agarose gel, stained with Ethidium bromide (10 mg/mL Sigma), for 60 minutes at 80V in 1xTE buffer. The DNA fragments were photographed and documented using Gel documentation system ChemiDoc XRS System from Biorad.

Table 2. SSR primer sequences

No	*Primers*	*Gene Bank accession number*	*Sequence of primer ('5—3')*	*Repeat motif*	*Expected (bp)/ Reference*
1	EST-SSR1	GD132221 CO910120	CCTTCCTAGCCACACACCTC GAAGGAATAACCGGCAGCTA	$(TC)_{10}$ $(TC)_5$	147 / [15]
2	EST-SSR2	GD056348 CA516988	ATTCTCTTCCACCGCCTTTT TCCGTTAAAGCACCATTTCC	$(CA)_{14}$ $(CA)_{10}$	143 / [15]
3	EST-SSR3	GD110370 BM062080	AAACGTCATCACAGCCATCA CGTAACGCACCCTCTAGGAA	$(CACCAT)_5$ $(CACCAT)_3$	159 / [15]
4	CAK6	CO908011 GD078211	GCATTCAAAGCCACACAAAA GACGGATAAGGAGGTGGTGA	$(GCC)_6$ $(GCC)_3$	141 / [15]
5	CAK21	GD119232 GD107906	TTGTTGGCCTGACTTTTGAA AAAATCGATCGTGGAGTTCG	$(ACA)_5$ $(ACA)_3$	163 / [15]
6	CAK35	CO909840 GD128267	TCCCACGAGTCTTTTTGAGG TCTGGTCTTCTTGGGAATCAA	$(AAACA)_6$ $(AAACA)_5$	230 / [15]
7	CAK58	CO776923 CK901965	GTGAACCACGAGCGGATTAT CGGAGTCAAAAGGACCTTCA	$(TTGGCA)_2$ $(TTGGCA)_1$	225 / [15]
8	EPM650	CO910134	CATGGGTGAGGGTACATGGT AGAGGGAAGGGTTATTTGCC	$(TA)_1$	251 / [16]

2.3. Data analysis

In the analyzed SSR loci, the frequencies of alleles in each category through all accessions were scored as either present (1) or absent (0). Initially, the potential of all the markers for estimating genetic variability was examined by measuring the marker informativeness through the counting of bands. Additionally, the primer banding characteristics such as number of scored bands (NTB), number of polymorphic bands (NPB) and percentage of polymorphic bands (PPB) were obtained. The discriminatory power of each SSR marker was determined by calculating the polymorphic information content (PIC) and heterozygosity (H) using the online PICcalc program [17].

PIC was calculated according to the formula [18]:

$$PIC = 1 - \sum_{i=1}^{l} p_i^2 - \sum_{i=1}^{I-1} \sum_{j=i+1}^{l} 2p_i^2 p_j^2$$

where,

pi and pj represent the population frequency of the ith and jth allele.

Heterozygosity (H) is a value that measures the genetic variation, calculated according to the formula:

$$H = 1 - \sum_{i=1}^{l} p_i^2$$

High heterozygosity values indicate that the crop may have evolved through long-term natural selection for adaptation or through historic mixing of strains of different populations. A low level of heterozygosity may be due to isolation with the subsequent loss of unexploited genetic potential [19].

In addition, the effective multiplex ratio (EMR), which is the product of the fraction of polymorphic bands and the number of polymorphic bands [20], was calculated using formula;

$$EMR = n\ x\ \beta$$

where,
n is the average number of fragments amplified by accession to a specific system marker (multiplex ratio)

β is estimated from the number of polymorphic loci (NPB) and the number of non-polymorphic loci (NMB); calculated by the following formula:

$$\beta = \mathrm{PB}/(\mathrm{NPB} + \mathrm{NMB})$$

Further, to analyze the stability and informativeness of all primers and to detect polymorphic loci among the genotypes the marker index (MI) [9], using the following formula:

$$MI = EMR\ x\ PIC$$

The resolving power (RP), the discriminatory potential of each primer was calculated as:

$$RP = \sum Ib$$

Ib represents the informative fragments, with a scale of 0/1,by the following formula;
Ib = 1 - (2 x |0.5 - pi|), where
pi is the proportion of accessions containing the ith band [21].

A dendrogram was constructed by using hierarchical clustering with UPGMA (Unweighted pair group method with arithmetic mean) analysis, where the genetic distance was measured based on Jaccard's dissimilarity index [22].The resulting tree was bootstrapped with 1000 replicates to obtain the best confidence [23]. For the complete data analysis, the DARwin 6.0.15 software was used [24].

3. Results and Discussion

In this study 14 pepper accessions, with a total of 9 EST SSR markers used (Table 2), amplified in total 25 bands. For each EST SSR primer, H, PIC, NPB, PPB, NMB, NSB, EMR, MI and RP were given at Table 3.

Table 3. Genetic diversity estimators for each primer

PRIMER	*H*	*PIC*	*NSB*	*NPB*	*NMB*	*PPB %*	*EMR*	*MI*	*RP*
EST-SSR1	0.83	0.8	2	1	1	50	0.571	0.46	16
EST-SSR2	0.79	0.75	4	3	1	67	1.286	0.96	12
EST-SSR3	0.8	0.76	4	4	0	100	2.143	1.63	15
EPMS650	0.53	0.55	4	4	0	100	1.429	0.79	9
CAK6	0.3	0.29	4	4	0	100	1.071	0.31	7
CAK21	0.83	0.8	2	1	1	50	0.500	0.40	26
CAK35	0.58	0.54	2	1	1	50	0.357	0.19	20
CAK58	0.490	0.48	2	2	0	100	1.143	0.55	16
Mean	0.64	0.62	3	2.5	0.5	77.1	1.06	0.66	15.06

H: Heterozygosity; PIC: Polymorphism information contents; NSB: Number of scored bands; NPB: Number of polymorphic band; NMB: Monomorphic bands; PPB: Percentage of polymorphic band; EMR: Effective multiplex ratio; MI: Marker index; RP: Resolving power

The SSR1, CAK21 and CAK35 resulted in 50% polymorphism; SSR2 has 67% polymorphic bands, whereas SSR3, EPMS650, CAK6 and CAK57 resulted in maximum, 100% polymorphic bands.

Average PIC values of all primers of SSR analysis were 0.62. The highest observed PIC value is 0.80 with SSR1 and CAK21.The average heterozygosity (H) value of the whole marker set was 0.64. These values ranged from 0.30 to 0.83. The lowest H value (0.3) was observed in CAK6, where the highest observed H value is seen in SSR1 and CAK21 (0.83). The highest value for the effective multiplex ratio (EMR) is seen with SSR3 primer (2.143) and the lowest value is observed in CAK35 primer (0.35), with an average EMR of 1.06 per primer. The highest discriminatory potential (resolving power-RP) value was 26, observed with the primer CAK21 and the lowest with value 7 is with CAK6 primer.

For the determination of primer usefulness, the MI (marker index) for EST- SSR primers was calculated. The SSR3 primer showed the highest MI of 1.63 and lowest MI was observed with CAK35, with an average MI of 0.66 per primer. The size of the amplified DNA fragments ranged from 100 bp to 330 bp and the amount of amplified DNA fragments ranged from 1 to 4 in all accessions, with the average number of bands per marker of 3 (Table2).

All EST-SSR primers generated products of the expected size [15, 16], in total 22 loci, including 18 loci that exhibited polymorphism and 4 loci that displayed monomorphism among the tested pepper cultivars. We observed two to three alleles per locus. Our results are in line with the results obtained from a study conducted in China in 2011, where the same set of primers resulted in 2 to 4 alleles per locus [15]. All the obtained results indicate that the genetic diversity among the pepper varieties showed sufficient dissimilarity characteristics, reflecting significant genetic diversity among all analyzed cultivars. The SSR1, CAK21 and CAK35 resulted in 50% polymorphism; SSR2 has 67%, whereas SSR3, EPMS650, CAK6 and CAK58 resulted in maximum, 100% polymorphic bands. It is reported that high diversity of locus is demonstrated in PIC value greater than 0.5 [25]. In our study, primers CAK6 and CAK58 had a polymorphic index content around lower than 0.55 while the remaining 4 primers had a PIC value higher than 0.53. The PIC value will be almost zero if there is no allelic variation and it can reach a max of 1.0 if a genotype has only new alleles [8].

The assessment of the discriminatory power of the given EST-SSR marker combinations, referred to as the resolving power (RP), were in the range of 7 to 26, with an average of 15.06. Marker CAK21 showed the highest RP value with 26. and the lowest was 7 with CAK6 marker. The total utility of each marker was assessed through the marker index (MI) factor, being a product of polymorphic information content and effective multiplex ratio [10]. Highest MI (1.63) is observed in SSR3 the lowest was with CAK35 marker (0.19). A high marker index indicates that the marker system used is useful for the chosen genetic population [26].

The fact that both EMR and MI were high in ISSR shows that the ISSR marker system is practical for molecular characterization of plants and determination of genetic relationships [27].

Based on the UPGMA Phenogram three main clusters were obtained. In Figure 1, the first cluster, separated into three subgroups, making up the largest group with 8 populations, with Chil, Corno Di Toro Roso (CD), clearly separated from all other varieties from cluster 1.

The second cluster is made up of 5 domestic pepper populations, including Istra pepper (IS), a regionally known hybrid. The third group, represented by Californian wonder (CW), being clearly evolutionary separated from all other taxa in the group. Numbers on branches correspond to bootstrap values of 1000 replications.

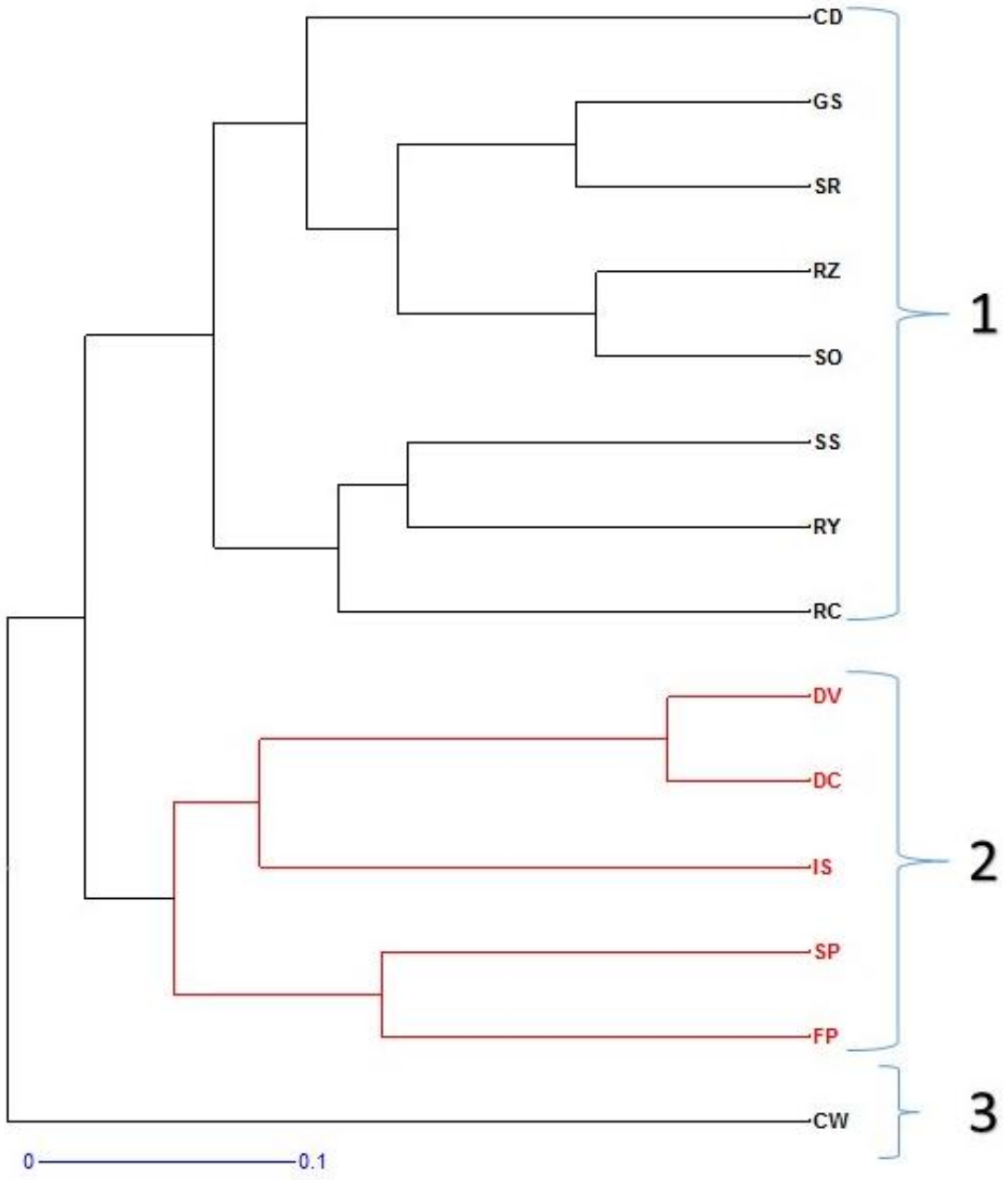

Figure 1. UPGMA dendrogram of local pepper cultivars based on genetic distance.

Labels: CW (Grossum, California wonder yellow), RY (Rotundum, Rotund žuta), RZ (Rotundum, Rotund zelena), RC (Rotundum, Rotund zelena), CD (Chili, Corno Di Toro Roso), SS (Kolgum, Slonova surla), SO (Somborka), SR (Soroskari), IS (Istra), GS (Giallo Dasti Roso), FP (Faruk paprika-domaca), SP (Srecko Peric paprika-domaca 2), DC (Domaca Cevas njive- domaca 3),DV(Domaca Visici-domaca 4). The line segment with the number 0.1 shows the length of branch that represents an amount genetic change of 0.1 nucleotide substitution per site.

Principle coordinate analysis (PCoA) was performed to further assess the genetic relationships among the pepper varieties by using 8 SSR markers, as seen in Figure 2.

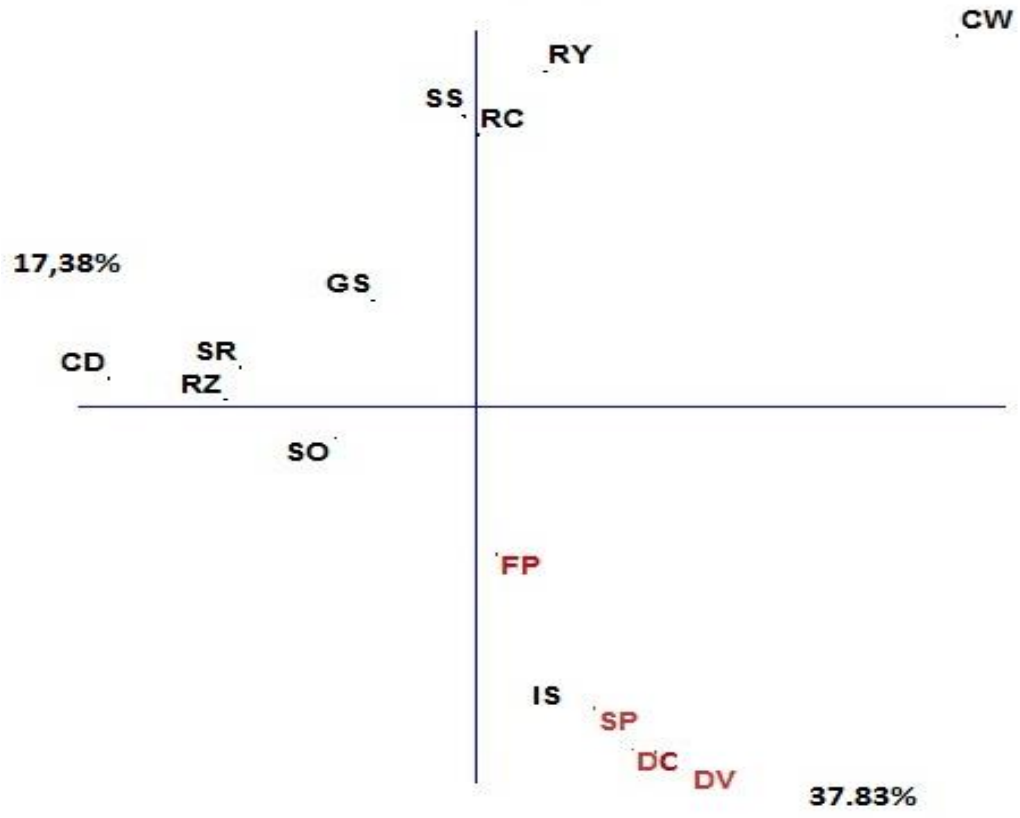

Figure 2. Principal coordinates analysis (PCoA) of *Capsicum annuum L* populations

Labels:CW (Grossum, California wonder), RY (Rotundum, Rotund žuta), RZ (Rotundum, Rotund zelena), RC (Rotundum, Rotund zelena), CD (Chili ,Corno Di Toro Roso), SS (Kolgum, Slonova surla), SO (Somborka), SR (Soroskari), IS (Istra), GS (Giallo Dasti Roso), FP (Faruk paprika-domaca), SP (Srecko Peric paprika-domaca 2), DC (Domaca Cevas njive-domaca 3),DV(Domaca Visici-domaca

The principal coordinate shows the populations IS, FP, SP, DC and DV which tend to form a discreet cluster. Neighbour-joining phenogram and PCoA of EST/SSR primers showed that all the domestic varieties and Istra pepper produced a set (cluster) together, being closest to each other. According to the phenogram and PCoA of EST/SSR, we can conclude that the samples farthest from each other were (CW and Domestic species). The close clustering of domestic landraces reveals the possibility that these landraces have a phylogenetic connection, although more genetic markers should be used to make this possibility statistically significant.

4. Conclusions

Eight EST-SSR microsatellite markers were used to assess the genetic diversity of common pepper cultivars from Bosnia and Herzegovina. The genetic diversity estimators showed sufficient dissimilarity characteristics and indicated significant genetic diversity among onion varieties. The phylogenetic analysis revealed that all domestic pepper varieties share same cluster and ancestors, indicating regional cohesion, whereas all onions in this study are clearly separated from Corno Di toso pepper, an Italian origin onion.
The obtained results provide valuable information to researchers for future conservation studies and new genetic insights on the specificity of local pepper varieties, focusing on domestic peppers, genetically distinct from all other analyzed hybrids.

Declaration of competing interest

The authors declare that they have no known financial or non-financial competing interests in any material discussed in this paper.

Acknowledgements

The authors wish to express their sincere thanks to UNDP Bosnia and Herzegovina, who has founded this project. In addition, our thanks go to Ibrahim Çelik for his advises during the calculations of primer indexes,

References

[1] Bosland P, Votava EJ and Votava EM. "Peppers: vegetable and spice capsicums", vol. 22, Cabi, 2012.

[2] Cronin, Joseph R. "The Chili Pepper's Pungent Principle: Capsaicin Delivers Diverse Health Benefits", *Alternative & Complementary Therapies*, vol. 8, no.2, p.110-113, 2002.

[3] Sanati, S., Razavi, B.M. and Hosseinzadeh, H.,"A review of the effects of Capsicum annuum L. and its constituent, capsaicin, in metabolic syndrome." *Iranian journal of basic medical sciencesv*. 21, no 5, 439, 2018.

[4] Hernández-Pérez, Talía, María del Rocío Gómez-García, María Elena Valverde, and Octavio Paredes-López."Capsicum annuum (hot pepper), "An ancient Latin-American crop with outstanding bioactive compounds and nutraceutical potential. A review", *Comprehensive Reviews in Food Science* and Food Safety, vol. 19, no.6, p. 2972-2993, 2020.

[5] Šutković, J., Glamočija, P., Karić, L. and Yildirim, A. "Genetic Characterization of Brassica oleracea L. var. acephala DC cultivars from Bosnia and Herzegovin"a. *Genetics & Applications*, vol. 5, no. 1, pp.40-50, 2021.

[6] Faostat, F.A.O, "Food and Agriculture Organization of the United Nations-Statistic Division", https://www.fao.org/faost at/en/# data, 2019.

[7] Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., Van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A. and Brettschneider, R, " Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories", *Molecular breeding*, vol. 3, no.5, pp.381-390, 1997.

[8] Guo, X. and Elston, "Linkage information content of polymorphic genetic markers", *Human heredity*, vol. 49, no.2, pp.112-118, 1999.

[9] Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A, "The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis", *Molecular breeding*, vol.2, no. 3, pp.225-238, 1996.

[10] Varshney, R.K., Graner, A. and Sorrells, M.E, Genetic microsatellite markers in plants: features and applications. *TRENDS in Biotechnology*, vol. 23, no.1, pp.48-55, 2005.

[11] Tsaballa, A., Ganopoulos, I., Timplalexi, A., Aliki, X., Bosmali, I., Irini, N.O., Athanasios, T. and Madesis, P, "Molecular characterization of Greek pepper (Capsicum annuum L) landraces with neutral (ISSR) and gene-based (SCoT and EST-SSR) molecular markers", *Biochemical Systematics and Ecology*, vol *59*, pp.256-26, 2015

[12]Gadaleta, A., Giancaspro, A., Zacheo, S., Nigro, D., Giove, S.L., Colasuonno, P. and Blanco, "A., Comparison of genomic and EST-derived SSR markers in phylogenetic analysis of wheat", *Plant Genetic Resources*, vol.9, no. 2, pp.243-246, 2011.

[13] Porebski, S., Bailey, L.G. and Baum, B.R," Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol component", *Plant molecular biology reporter*, *15*(1), pp.8-15, 1997.

[14] Fischer, D. and Bachmann, K, "Onion microsatellites for germplasm analysis and their use in assessing intra-and interspecific relatedness within the subgenus Rhizirideum". *Theoretical and Applied Genetics*, vol. *101*, no.1, pp.153-164, 2000.

[15] Kong, Q., Zhang, G., Chen, W., Zhang, Z. and Zou, X, "Identification and development of polymorphic EST-SSR markers by sequence alignment in pepper, Capsicum annuum (Solanaceae)", *American Journal of Botany*, vol. *99*, no.2, pp.59-61, 2012.

[16] Portis, E., Nagy, I., Sasvári, Z., Stágel, A., Barchi, L. and Lanteri, S, "The design of Capsicum spp. SSR assays via analysis of *in silico* DNA sequence, and their potential utility for genetic mapping", *Plant Science*, vol. *172*, no.3, pp.640-648, 2007.

[17] Nagy, S., Poczai, P., Cernák, I., Gorji, A.M., Hegedűs, G. and Taller, J, "PICcalc: an online program to calculate polymorphic information content for molecular genetic studies" *Biochemical genetics*, *vol. 50, no.9,* pp.670-672, 2012.

[18] Serrote, C.M.L., Reiniger, L.R.S., Silva, K.B., dos Santos Rabaiolli, S.M. and Stefanel, C.M, "Determining the Polymorphism Information Content of a molecular marker" *Gene*, *vol.726,* p.144175. 2020.

[19] Liu, Ben Hui. Statistical genomics: linkage, mapping, and QTL analysis. CRC press, 2017.

[20] Kumar, M., Mishra, G.P., Singh, R., Kumar, J., Naik, P.K. and Singh, S.B, "Correspondence of ISSR and RAPD markers for comparative analysis of genetic diversity among different apricot genotypes from cold arid deserts of trans-Himalayas."*Physiology and Molecular Biology of Plants*, vol. *15, no. 3,* pp.225-236. 2009.

[21] Prevost, A. and Wilkinson, M.J,"A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars", *Theoretical and applied Genetics*, vol. 98, no.1, pp.107-112, 1999.

[22] Real, R. and Vargas, J.M, "The probabilistic basis of Jaccard's index of similarity", *Systematic biology*, vol. *45*, no.3, pp.380-385,1996.

[23] Felsenstein, J," Confidence limits on phylogenies: an approach using the bootstrap" *evolution*, vol.*39*, no.4, pp.783-791, 1985.

[24] Karić, L., Golzardi, M., Glamočlija, P. and Šutković, J, "Genetic diversity assessment of Allium cepa L. cultivars from Bosnia and Herzegovina using SSR makers" *Genetics and Molecular Research*, vol.*17*, no.1, 2018.

[25] Botstein, D., White, R.L., Skolnick, M. and Davis, R.W, "Construction of a genetic linkage map in man using restriction fragment length polymorphisms", *American journal of human genetics*, vol.*32, no.3*, p.314, 1980.

[26] Ghanbari, A., Faraji, M., Behnamian, M., Estaji, A., Pyrayesh, A. and Fahim, S, "Genetic diversity evaluation of some walnut (Juglans regia L.) genotypes in Meshkin-Shahr by ISSR marker " *Journal of Nuts*, vol.*10*, no.1, pp.1-8., 2019.

[27] Singh, A., Dikshit, H.K., Jain, N., Singh, D. and Yadav, R.N, "Efficiency of SSR, ISSR and RAPD markers in molecular characterization of mungbean and other Vigna species", 2014.