Genetic diversity assessment of *Allium cepa* L. cultivars from Bosnia and Herzegovina using SSR makers

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ABSTRACT. The five most common cultivars from the genus *Allium cepa* L. in Bosnia and Herzegovina (BiH) were analysed, focusing on Konjic onion. Seven SSR markers for genetic similarity analysis were used to address the genetic backgrounds. The polymorphic relationship between the SSR markers was analyzed by using Polymorphic Information Content (PIC) and Heterozygosity (H) by using the online available PICcalc program. For the complete data analysis and phylogenetic tree construction, the DARwin 6.0.13 software was used. The total number of obtained SSR alleles was 30 bands, where 56.7% were polymorphic with the range of allele size of 130 to 650 base pairs (bp). The mean polymorphic information content (PIC) was 0.435 and the expected heterozygosity (H) values ranged from 0 to 0.785. Jaccard's coefficient of similarity values ranged from 0.14 to 0.55. The results in this study represent the first genetic diversity data on the onion cultivars in BiH and show significant dissimilarity among the onion cultivars. This study confirmed that the molecular SSR analysis represents an efficient tool for *Allium cepa* L. landrace genetic similarity analysis.

Key words: *Allium cepa* L; SSR marker; Genetic characterization; Polymorphism; Heterozygosity

INTRODUCTION

Molecular genetic markers, as Restriction Fragment Length Polymorphism (RFLP), Random Amplifies Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP) and Simple Sequence repeats (SSR), are most frequently used in the characterization and evaluation of genetic diversity within and between species and populations (Guo-Liang Jiang, 2013). However, the most used types of genetic markers are microsatellites or simple sequence repeats (SSR). SSRs are motifs that occur randomly in the coding and noncoding part of the genome sequence (Varshney et al., 2005).

Microsatellite markers are widely used in plant, human, and animal species due to their wide range of applications, mostly based on a high degree of intraspecific polymorphism, greater reliability, co-dominant genetics and greater reproducibility compared to other molecular DNA markers (Powell et al., 1996; Jones et al., 1997).

Several sets of SSR markers have been previously used to detect genetic diversity in onion germplasm (Hanci and Gökçe, 2016; Jakse et al., 2005; Chakraborty et al., 2015; Simó et al., 2014; Fischer and Bachmann, 2000), but no studies including onion genotypes from South East European countries, including Bosnia and Herzegovina, have been recorded so far.

Onion is the most commercially important vegetable crop of the *Alliaceae* family. In Bosnia and Herzegovina (BiH) it is the fourth most important vegetable crop. Over 5, 000 ha of onions are planted annually, with average yield between 7 and 9 tons/ha (Muratović and Crnica, 2011). Onions are generally produced manually on small non-irrigated plots and there has been a steady annual increase in the area sown with onions (Subotić, 2011). Onions in BiH are mostly produced from onion sets (small dry onion bulbs produced the previous year). According to our market analysis, 4 varieties for decades dominate local seeds markets; Dutch yellow, Stuttgarter riesen, Majski srebrenjak and Ptujska rdeća. Due to the poor economic situation in BiH some farmers are using the onion set of Konjic onion, autochthonous onion cultivar of unknown origin. Konjic onion is a cultivar from the Buturović polje, Municipality of Konjic. Farmers from Buturović polje claim to have been using this cultivar for more than 70 years. Konjic onion bulb is morphologically classified as "large", with the average bulb size ≤100 g, and a flat globe shape with yellow skin colour (Vukašinović et al., 2005). Its flavour is pungent, with an average dry matter content of ≤ 14%, 1.5% of total proteins and 9.5% of total sugars (Čota et al., 2013). Konjic onion has, on average, between a 24.5% and 51% better yield compared to the Stuttgarter riesen, a standard cultivar in BiH (Čota et al., 2015). In the 90s, the annual level of production of onion exceeded 100 tons. However, it is estimated that the current production is below 20 tons per year. One of the main reasons for the decrease in production is the due to the fact that this variety is not listed in BiH Common List of Varieties (Sortna lista BiH, 2017).

The biodiversity of crops landraces, and their wild relatives, represents a valuable genetic resource in modern breeding techniques. In the last 100 years, about 75% of the biodiversity was lost because of the farmers' transition from local varieties and landraces to modern cultivars (Thrupp, 2003). In this regard, modern breeding employs marker analysis of wild crop relatives to identify possesses genetic variation lost during the domestication process. Simple Sequence repeats (SSR) are considered as one of the most powerful DNA based markers, having great advantage in genetic cross-species comparisons (Zhu et al., 2016). This is not only because of their high reproducibility and co-dominance inheritance, but also because of their multi-allelic character and extensive genome coverage (Varshney et al., 2005).

The aim of this study was to assess the use of SSR markers sets, proposed by Fischer & Bachmann, 2000; Masuzaki et al., 2006 and Simó et al., 2014, for genetic characterization of the local onion landraces from BiH. The origin of Konjic onion is unknown, therefore we included these candidates, which could have genetically contributed to the process of crossbreeding.

MATERIALS AND METHODS

We used 5 onion cultivars in this study; Ptujska rdeća (Sjemenarna d.o.o., Ljubljana, Slovenia) Majski Srebrenjak (Green Garden, Italy), Dutch yellow (Green Garden, Dutch), Stuttgart riesen (Marmix-BiH, Germany), also known as Stuttgarter giant (Friedman and Rubin, 2015) and Konjic onion (farmers Buturović polje, Municipality of Konjic, BiH). All of the analysed cultivars, except the Konjic onion, are commercially available onion varieties. Onion seeds for commercial cultivars were obtained from local agro-store (Sjemenarna d.o.o. Široki Brijeg, BiH). The Konjic onion was collected from farmers, located in Buturović polje, Municipality Konjic from Bosnia and Herzegovina, as fresh bulbs and stored in -20°C until DNA isolation.

Genomic DNA isolation

Genomic DNA was isolated from the frozen leaves and dry seeds which were ground into fine powder using liquid nitrogen. The DNA was extracted according the modified CTAB (Cetyltrimethyl ammonium bromide) protocol (Porebski et al., 1997). The DNA quality was analysed on 1% agarose gel and quantified using a Multiscan GO (Thermo Fisher Scientific, USA) and stored in 100µl aliquots in 1XTE buffer at –20°C until PCR amplification.

SSR analysis

The polymorphic SSR markers used in this study are listed in Table 1 (8, 9.18). All PCR amplifications were performed in thermal cycler (BioRad C1000, USA). The PCR reactions were performed in 25 µl total volume, containing 0.70 µl of 0.2 mM dNTPs, 3 µl of 2.5 mM $MgCl_2$, 4 µl of 10× Taq buffer, 0.2µl of Taq DNA polymerase (Sigma Aldrich, 5 units/µl), 1µl of each primer (50 ng/µl, Sigma Aldrich, Germany), 20ng of template DNA (5µl) and remaining dH_2O.

The PCR protocol included two phases, starting with an initial denaturation phase at 95°C for 5 min. The first phase was 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 & Bachmann, 2000, 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), for 60 minutes at 80V in 1x TE buffer. The DNA fragments were photographed and documented using a Gel documentation system (Chemi Doc XRS System, BioRad, USA).

Data analysis

In the analysed SSR loci, the frequency of alleles in each category through all samples was 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 bands counting. 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 PIC_{calc} program (Nagy et al., 2012)

PIC was calculated according to the formula (Hildebrand et al., 1992):

$$PIC = 1 - \sum_{i=1}^{i} pi^2 - \sum_{i=1}^{i-1} \sum_{j=i+1}^{i} 2\, pi^2\, pj^2$$

where,

- p_i and p_j represents the population frequency of the i^{th} and j^{th} allele.

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

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

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 (Liu, 1997).

In addition, the effective multiplex ratio (EMR), which is the product of the fraction of polymorphic bands and the number of polymorphic bands (Kumar et al., 2009), 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:

$$\beta = PB / (NPB + NMB)$$

Further, to analyze the stability and in formativeness of all primers and to detect polymorphic loci among the genotypes the marker index (MI) was calculated, using the following formula (Powell et al., 1996):

MI=EMR × PIC

The resolving power (RP), the discriminatory potential (Prevost and Wilkinson, 1999) of each primer was calculated as:

$RP + \sum IB$, where

- *Ib* represents the informative fragments, with a scale of 0/1, by the following formula;

Ib = 1 - (2 × |0.5 - pi|), where

- *pi* is the proportion of accessions containing the i^{th} band

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 (Real and Vargas, 1996). The resulting tree was bootstrapped with 1000 replicates to obtain the best confidence (Sanderson, 1989). For the complete data analysis, the DARwin 6.0.13 software was used (Perrier and Jacquemoud, 2006).

RESULTS

The seven SSR markers used in this study amplified in a total of 30 bands (Table 1). All the markers showed clear and observable bands (Figure 1). The average PIC values of all primers of SSR analysis was 0.435. The highest observed PIC value was 0.768 and the lowest with AMS8 and AMS14 primers. The average heterozygosity (H) value of the whole marker set was 0.455. These values ranged from 0 to 0.785.

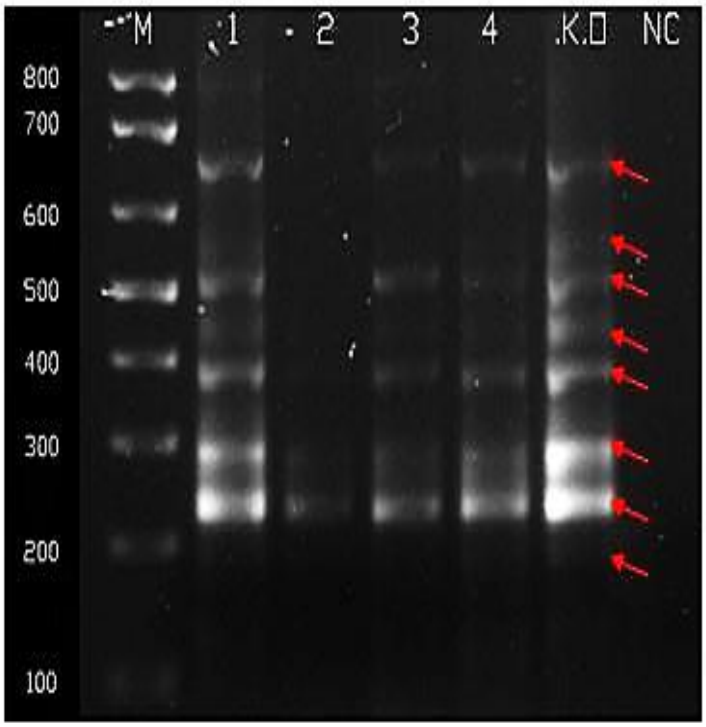

Figure 1. SSR profile of different genotypes generated by primer AMS12 on 3% agarose gel (From left to right, M (Direct Load™ PCR 100 bp Low Ladder), 1=Ptujska rdeća, 2=Majski srebrenjak, 3=Dutch yellow, 4=Stuttgarter riesen, K. O=Konjic onion. NC=Negative control. The red arrows indicate accounted bands in 5^{th} line (K.O)

The lowest H value (0) was observed in AMS08 and AMS14, where the highest observed H value is seen in AMS16 (0.785) and AMS12 (0.769) (Table 2).

In addition, for each AMS primer, PPB, NMB, NSB, EMR, MI and RP were calculated as given in Table 1.

Table 1. Summary statistics of *Allium cepa L*. SSR markers (n = 30)

Primers	Primer sequence ('5—3')	Repeat motive	Size range (bp)	PIC	H
AMS08	F:GCCACGATGTTGAGATTTCG R:CCCGAATATCCCACCAGTTC	(CTT)3 T (CTT)14 TT (CT)2 TCT	205	0	0
AMS10	F:TTCATGTTGTATTGAGATTTGG R:GAAGGAATGGAAGCAGTTC	(AT)4 (GT)16	140-300	0.411	0.381
AMS12	F:AATGTTGCTTTCTTTAGATGTTG R:TGCAAAATTACAAGCAAACTG	(CA)25	190-650	0.726	0.769
AMS14	F:CCCCTGAGTAAATTCAAAATCC R:TCCTTAGTATAATTTCGGGGTAAC	(CA)28 (TA)4	130, 190	0	0
AMS16	F:CTGCATTAAAACAACCAAACTTG R:GAGCTCCACTTCTTCCAAACTAG	(CA)20 (TA)2	250-270	0.785	0.751
AMS25	F:GAGGGCAGTGTTAGCATTCC R:GAGCTCCACTTCTTCCAAACTAG	(AC)21 (AT)3	180-510	0.751	0.785
AMS30	F:CACTAATGGGGTAAATAATGTTCTAC R:TTGCCTTGAAATCCAGAC	(CA)8 CG (CA)22 (TA)4	190-340	0.375	0.500

n=Test population size; N_A = Number of alleles for each primer (range); H= Heterozygosity; PIC=Polymorphism Information Content.

All primers, except AMS08 and AMS14, resulted in 0% polymorphic bands while the AMS10 and AMS12 showed the highest polymorphic behaviour with 66.7% and 80%, respectively. The effective multiplex ratio (EMR) results for this study showed the that highest effective multiplex ratio (EMR) of 5.6 in primer AMS12, and the lowest effective multiplex ratio is observed in primers AMS08 and AMS14 with 0.0, an average EMR of 1.63 per primer.

The discriminatory potential (resolving power) of each primer primers was calculated. The highest RP value was 10, observed with AMS08 and AMS14 primers and the lowest was 5.6 with AMS10 primer.

To determine the general usefulness of the system of primers used, the MI (marker index) for each SSR primer was calculated as a product of polymorphic information content and effective multiplex ratio (Powell et al., 1996). Highest MI (4.16) was for AMS12 and lowest (0.0) was for AMS08 and AMS14 primers. The average RP and MI values of all primers are 7.60 and 1.20, respectively.

The size of the amplified DNA fragments ranged from 130 bp to 650 bp and the amount of amplified DNA fragments ranged from 1 to 10 in all accessions, with the average number of bands per marker of 4.3 (Table 2 and Figure 1). When compared with Spanish, Iranian and Japanese onion, the SSR markers in the domestic variety, Konjic onion, showed different number of alleles per locus (Table 3).

Table 2. Genetic diversity estimators for each primer in *Allium cepa L*. varieties

PRIMERS	NSB	NPB	NMB	PPB%	EMR	MI	RP
AMS08	1.0	0.0	1.0	0.0	0.0	0.00	0
AMS10	6.0	4.0	2.0	66.7	2.5	1.10	6.3
AMS12	10.0	8.0	2.0	80.0	5.6	4.65	7
AMS14	2.0	0.0	2.0	0.0	0.0	0.00	0
AMS16	4.0	2.0	2.0	50.0	1.4	0.79	7
AMS25	5.0	2.0	3.0	40.0	1.4	0.60	7.2
AMS30	2.0	1.0	1.0	50.0	0.8	0.38	4

NSB: Number of Scored Bands; NPB: Number of Polymorphic Bands; NMB: Monomorphic Bands;
PPB: Percentage of Polymorphic Band; EMR: Effective Multiplex Ratio; MI: Marker Index; RP: Resolving power

Table 3. SSR polymorphic band detection by electrophoresis in target onion compared onion from Japan, Iran, and Spain.

Microsatellite	Expected size (bp)[1]	Observed allele size (bp) in Konjic onion	Observed allele number			
			K. O[2]	Japan[3]	Iran[4]	Spain[5]
AMS08	205	205	1	3	3	9
AMS10	157	160, 180, 200, 300	4	3	3	8
AMS12	274	190, 250, 300, 330, 390, 430, 500, 520, 650	9	2	5	11
AMS14	169	130, 190	2	1	10	14
AMS16	261	250, 270	2	2	5	8
AMS25	235	180, 200, 300, 510	4	2	1	9
AMS30	342	340	1	1	6	12

[1] Fischer & Bachmann 2000, [2] Konjic onion (K.O) allele number, [3] Masuzaki et al., 2006, [4]Nafchi et al., 2012, [5]Simóet al., 2013

Based on the dendrogram obtained from SSR profiles, representing a rooted tree, two main clusters were obtained (Figure 2).

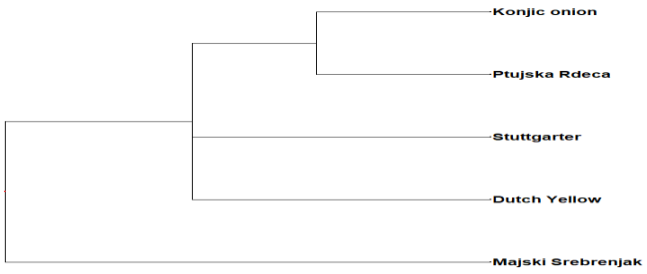

Figure 2. Dendrogram of onion cultivars based on genetic distance obtained from SSR markers revealed by UPGMA method.

Group one represents the out-group with Majski Srebrenjak, clearly separated from all remaining accessions. The second group is made of two sub-clusters, Dutch yellow and Stuttgarter riesen in one sub-cluster and Konjic onion and Ptujska rdeća making up the second sub-cluster of group two.

DISCUSSION

In our study, genetic diversity of five most common Bosnia and Herzegovina (BiH) onion genotypes, including the sample of unknown origin, were evaluated by the SSR markers. Results indicating genetic diversity showed sufficient dissimilarity characteristics and reflecting significant genetic diversity among onion cultivars. Genetic variation among 5 onion accessions was assessed with the 7 SSRs loci and a total of 30 alleles were detected, out of which 17 bands (56.7%) were polymorphic. It is reported that high diversity of locus is demonstrated in PIC value greater than 0.5 (Botstein et al., 1980). In our study, the samples with two primers showed no polymorphism rate (AMS08 and AMS14), two primers (AMS10 and AMS30) had insufficient polymorphic index content while the remaining 3 primers (AMS12, AMS16 and AMS25) had a PIC value higher than of 0.7. 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 allele (Guo, and Elston, 1999). Therefore, only 3 primers used in this study were informative, sufficient for distinguishing the polymorphic rate of all markers at specific loci. Four of the SSR primers (AMS10, AMS12, AMS16 and AMS25) have high RP values (from 6.3 to 7.2), hereof being the most informative primers for distinguishing these onion genotypes in this study. However, the resolving power provides no information on the ability of a primer to reflect the genetic or taxonomic relationships of a group of genotypes under the study (Prevost and Wilkinson, 1999). The average MI was 1.1, primer AMS08 and AMS14 with 0 and primer AMS12 with 4.65.

The dendrogram generated using UPGMA cluster analysis grouped 5 genotypes into two major clusters with Jaccard's dissimilarity index ranging from 0.14 to 0.55, with threshold equality of 100%. When sample of unknown origin is used together with potential parents, proposed primers are sufficient to reveal wide genetic variability among the genotypes. Majski Srebrenjak, as an alone taxon, is clearly separated from other group, indicating its diversity in the onion genetic pool, diverted through evolutionary history. The clustering dendrogram divided Ptujska rdeća and Konjic onion into a separate sub-cluster, making them closely related and clearly evolutionary separate from other clusters. Lastly, similarity is also observed between Stuttgarter and Dutch yellow that are placed into the same cluster. Each group shares a single common ancestor. Dutch yellow is known to be a close strain of the Stuttgarter risen (Dutch Onions, 2016), as confirmed by our analysis. In addition, it is expected that the Ptujska rdeća onion and Konjic onion are genetically similar since they are regionally connected, both located in the Balkans, indicating that both sub-clusters are in accordance with the known geographical location. Further, the SSRs markers used in this study showed, in total, a higher percentage of amplification than in previous reports (Masuzaki et al., 2006; Nafchi et al., 2012). We have purposely chosen seven microsatellite markers in order to compare the results with distantly related onion species from Iran, Spain and Japan. In Japan onion cultivars AMS12 primer resulted in bands between 237bp and 274bp Masuzaki et al., 2006 and bands between 220bp and 430bp were obtained in Iranian onion cultivars, as seen in Table 3 (Nafchi et al., 2012). We observed the allele size between 190bp and 650bp in the Bosnian onion accessions using the same marker. Such different values reflect the diversity and genetic variations originating from different regions of the world (Jakse et al., 2005; Solmaz et al., 2016; McCallum et al., 2008; Mitrova et al., 2015; Mahajan et al., 2009).

CONCLUSION

SSR microsatellite markers were used to assess the genetic diversity of onion cultivars from Bosnia and Herzegovina. The obtained results confirmed the efficiency of SSR markers. The average PIC and H values of all SSRs (0.435; 0.455) were in range in report of similar studies on the same marker sets. The remaining estimators for genetic diversity showed sufficient dissimilarity characteristics and indicates significant genetic diversity among onion varieties. The phylogenetic analysis revealed that the Konjic onion shares similarity with the Ptujska rdeća, indicating regional cohesion, whereas all onions in this study are clearly separated from the Majski Srebrenjak onion, an Italian origin onion. The molecular genetic analysis performed in this study provides valuable information to researchers for future conservation studies new genetic insights on the specificity of local onion varieties. The obtained results provide support to local farmer's maintaining of Konjic onion cultivar and provide argument to the government bodies to add this variety to the BiH Common List of Varieties as a part of the effort to conserve this autochthonous variety.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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