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Original scientific paper

GENETIC STRUCTURE OF APPLE ACCESSIONS MAINTAINED *EX SITU* IN BOSNIA AND HERZEGOVINA EXAMINED BY MICROSATELLITE MARKERS

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In order to identify possible mislabeling of the apple accession maintained *ex situ* in Srebrenik and to gain insight into the genetic structure of the conserved germplasm, 14 accessions from the collection were genotyped using 10 SSR (Simple Sequence Repeats) markers. Obtained SSR profiles were then added to an existing database constructed for previously characterized 24 traditional and 13 international, reference apple cultivars maintained at the same collection. Bayesian analysis implemented in the STRUCTURE program grouped 42 out of 51 analyzed apple accessions (38 traditional and 13 international) into three RPPs (reconstructed panmictic populations) with probability of membership *qI* higher than 75%. Almost all international, reference cultivars grouped in RPP3, whereas traditional B&H cultivars from the Srebrenik collection grouped in all three RPPs. Large and significant differentiations between all three individual RPPs were detected through the analyses of molecular variance and confirmed with FCA (factorial correspondence analyses). NJ cluster analysis, based on the Bruvo genetic distance, revealed that out of 38 traditional B&H apple cultivars, analyzed in the study, 'Ljepocvjetka', 'Bobovec' and 'Bobovec J' grouped closest to the international reference cultivars. Available date indicates that unlike a large number of B&H apple cultivars which were introduced during the reign of the Ottoman Empire, 'Ljepocvjetka' and 'Bobovec' were probably

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introduced at a later date. Cluster analyses also enabled the detection of one synonym and three homonyms within the collection. In four cases, previously conducted identification based on phenotypic analyses was confirmed by genetic analyses. Results of the structure analyses indicate a heterogeneous genetic structure of the analyzed accessions. This characteristic of the B&H apple germplasm could be useful for future breeding programs.

Key words: traditional apple cultivars, SSR, factorial correspondence analyses, Bayesian analyses

INTRODUCTION

With the aim of conserving fruit genetic resources in Bosnia and Herzegovina (B&H) an apple and pear *ex situ* collection was established in Srebrenik (northeast Bosnia), in year 2000. Accessions maintained at the collection were gathered from a wide range of locations in Bosnia and Herzegovina. During the collecting missions, a large set of traditional knowledge, including cultivar names, for many of the genotypes was also gathered from farmers maintaining the apple and pear trees. However, in some cases the names obtained from the farmers were descriptive in nature and generally unreliable. Alongside the collected B&H apple germplasm, in 2001 fifteen international, commercial apple cultivars were planted within the *ex situ* collection in Srebrenik. These genotypes have in mean time been regularly used as reference cultivars in studies on the B&H traditional apple germplasm. Results of phenotypic analyses carried on both traditional and reference materials indicate a significantly higher diversity for all morphological traits within the B&H traditional apple germplasm (GASI *et al.,* 2011). Observed phenotypic data, especially pomologic, was used for the identification of accessions for which the curators did not obtain reliable names during the collecting mission. However, a recent review by NYBOM and WEISING (2010) reports that DNA markers reveal much higher number of mislabeled plant accessions (typically 25–30% mislabeling) when compared to traditional pomological characterization (typically 5–10% mislabeling). Aside for correct identification of the accessions, genetic markers can assist in the assessment of the genetic diversity of a certain apple germplasm (MARIĆ *et al*., 2010) as well as to evaluate commercial traits such as ethylene production in apple fruit (MARIĆ *et al*., 2005; 2007). Among a large number of different DNA molecular markers, Simple Sequence Repeat (SSR) markers, also known as microsatellites, due to their abundance, reproducibility and polymorphism are highly useful (NYBOM and WEISING, 2010), especially for examining genetic diversity. Microsatellites have previously been used for genetic assessment of 24 traditional and 13 international apple accessions maintained at the *ex situ* collection in Srebrenik (GASI *et al*., 2010). Traditional accessions selected for genotyping in that study were some of the best known old B&H cultivars, for which the curators found and documented large amounts of traditional knowledge. SSR markers have also been used to examine the genetic structure of B&H apple germplasm found on farms in Bosnia and Herzegovina (GASI *et al*., 2013). Similar studies on genetic structure of local apple germplasm maintained on farm and *ex situ* have previously been carried out by PEREIRA-LORENZO *et al*. (2008) and URRESTARAZU *et al*. (2012) in Spain.

In order to solve issues regarding the mislabeling of the apple accession maintained *ex situ* in Srebrenik, as well as to gain insight into the genetic structure of the germplasm conserved in this collection, additional genetic characterization needs to be undertaken. Therefore, the objectives of this study were: 1) to add the genetic profiles of 14 apple accessions, maintained at

the *ex situ* collection in Srebrenik, to a database constructed for previously characterized 37 traditional and international apple cultivars from the same collection using a set of 10 SSR markers and 2) to examine the genetic structure of 51 apple accessions maintained *ex situ* in Bosnia and Herzegovina.

MATERIALS AND METHODS

A total of 14 traditional B&H apple accessions (Table 1) maintained at the *ex situ* collection in Srebrenik were genotyped with a set of 10 SSR markers, previously used by GASI *et al*. (2010) on 37 selected apple accessions (24 traditional and 13 international, reference cultivars) from the same collection. Unlike the previously characterized 24 traditional apple accessions, whose names were gathered during the collecting missions from the farmers cultivating them, 14 apple accessions analyzed in this study have been assigned names based on a morphologic identification conducted by curators of the collection.

Table 1. Fourteen apple accessions maintained at the ex situ collection in Srebrenik analyzed in this study using 10 SSR markers. Accessions names assigned based on a morphologic identification conducted by curators of the collection. In cases where the same genotyped has already been registered within the collection, a number two or three is added to the name of the supposed synonym.

Acc. number	Assigned name
1.	Kožara
2.	Mirisavka
3.	Masnjača
4.	Senabija 2
5.	Žuja 2
6.	Pašinka
7.	Stana
8.	Kolmana
9.	Francuska Kožara
10.	Kanjiška
11.	Funtača 2
12.	Habikuša 2
13.	Habikuša 3
14.	Srebrenička 2

SSR analyses

Tissue samples for DNA analyses were collected in the spring of 2011, from a single tree for each accession. DNA extraction was performed with Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, California, USA) according to the protocol included in the kit. Ten primer pairs used for SSR amplifications have been previously published by GIANFRANCESCHI *et al*.

(1998) and LIEBHARD *et al*. (2002). PCR amplification of SSR sequences was performed in a Veriti TM Thermal Cycler (Applied Biosystems, Foster City, California, USA) using fluorescent labeled primers, which enabled the detection of PCR products using ABI 3130 Genetic Analyzer (Applied Biosystems). All PCR amplifications were performed as described in GIANFRANCESCHI *et al*. (1998). The PCR product was diluted with ddH_20 (1:50), then added to 8.75 µl HiDi and 0.25 µl Genescan 500 LIZ size standard. Obtained data was analyzed using the software package GeneMapper 4.0 (Applied Biosystems).

Biostatistical analyses

SSR profiles obtained by genotyping 14 accessions from the *ex situ* apple collection in Srebrenik were added to the existing microsatellite database constructed in a previous study on apple accessions maintained at the same collection.

Population genetics software SPAGeDI 1.2 (HARDY and VEKEMANS, 2002) was used for calculating allele frequencies, gene diversity (NEI, 1978) and Fst (WEIR and COCKERHEIM, 1984). Analyses of molecular variance (EXCOFFIER *et al*., 1992), based on the stepwise mutation model (OHTA & KIMURA, 1973), was performed using GenoType software with 1000 permutations. Genetic distance between accessions (BRUVO *et al*., 2004), based on a two-phased mutation model (DI RIENZO *et al*., 1994) was calculated using GenoDive software. Both programs are part of the GenoType/GenoDive package (MEIRMANS and VAN TIENDEREN, 2004).

In order to examine population structure we used the Bayesian model-based cluster procedure within STRUCTURE version 2.2.3 (PRITCHARD *et al*., 2000). Method described by EVANNO *et al*. (2005) was used to estimate the most probable K value for the analyzed data. This was done through Structure harvester ver. 0.6. application (EARL and VONHOLDT, 2011). Assignment of one cultivar in a RPP (reconstructed panmictic populations) was provided by a probability of membership *qI* chosen at 75%.

Neighbor-joining cluster analysis, based on the above mentioned genetic distance was performed in MEGA 5 software (Molecular Evolutionary Genetics Analysis), (TAMURA *et al*., 2011). A multivariate analyses, FCA (factorial correspondence analysis) based on allele frequencies was performed using Genetix 4.02 (BELKHIR *et al*., 2001), which meant excluding the triploid genotypes.

RESULTS AND DISCUSSION

SSR polymorphism

Overall, ten SSR primer pairs amplified 113 alleles or on average 11.3 alleles per analyzed loci (Table 2). Compared to the previous study on selected apple genotypes maintained at the *ex situ* collection in Srebrenik (10.4) (GASI *et al*., 2010) this represents a slight increase. However, higher values have been reported by GASI *et al*. (2013) (13.5), who examined B&H apple genetic resources maintained on farms in Sarajevo and eastern Bosnia. PEREIRA-LORENZO *et al*. (2008) who studied local Spanish cultivars also obtained somewhat higher number of alleles per loci (12.5). Much higher values have been reported by URRESTARAZU *et al*. (2012) (16.69) and VAN TREUREN *et al*. (2010) (18.5), on apple germplasm collected in northeastern Spain and Holland. However, number of accessions analyzed in both studies was above 500. Regarding the value for gene diversity (NEI, 1978), obtained in this study (0.79) (Table 2), it was very similar to the value reported by GASI *et al*. (2010) (0.78), URRESTARAZU *et al*. (2012) (0.82)

and GASI *et al*. (2013) (0.80), but somewhat higher than what was reported by PEREIRA-LORENZO *et al*. (2008) (0.73).

Table 2. Allele size range (bp) for all the analyzed apple accessions, number of alleles per locus and gene diversity (NEI, 1978), based on 10 SSR loci, for 51 apple accessions from the ex situ collection in Srebrenik, as well as for each of the three reconstructed populations (RPPs) defined by Structure (PRITCHARD et al., 2000).

	All analyzed accessions (N=51)			RPP1 (N=11 *qI*>75%)		RPP2 (N=11 *qI*>75%)		RPP3 (N=20 *qI*>75%)	
Locus code	Size range (bp)	No. of alleles	Gene diversity	No. of alleles	Gene diversity	No. of alleles	Gene diversity	No. of alleles	Gene diversity
CH01H01	111/145	9	0.86	6	0.83	6	0.74	6	0.82
CH05E03	153/193	14	0.87	7	0.82	6	0.8	9	0.89
CH05E04	149/172	9	0.82	6	0.72	6	0.65	6	0.75
CH01H02	235/251	8	0.75	5	0.52	6	0.82	7	0.73
CH02C02a	130/192	20	0.92	9	0.88	7	0.84	13	0.89
CH04E02	139/166	9	0.56	4	0.57	3	0.39	6	0.66
CH01H10	93/119	12	0.81	7	0.88	6	0.68	6	0.75
CH02D08	212/254	11	0.84	6	0.75	5	0.78	8	0.86
CH02C02b	102/123	6	0.6	3	0.52	3	0.62	4	0.55
CH02C06	218/263	15	0.91	7	0.84	7	0.87	9	0.85
Mean		11.3	0.79	6	0.73	5.5	0.72	7.4	0.78

Genetic structure

Bayesian analysis, done within Structure, was based on 10 SSR loci and included 51 apple accessions (38 traditional B&H and 13 international). Subsequent ΔK analyses (EVANNO *et al*., 2005) revealed that K=3 was the most probable one (Fig. 1). The first RPP included 14 accessions, eleven of them with *qI* greater than 75%. The second RPP included 11 accessions, all of them with *qI* greater than 75%. The third RPP included 26 accessions, twenty of them with *qI* greater than 75%. Bayesian analyses revealed that all but one ('Piros') of the international reference cultivars grouped in RPP3, whereas traditional B&H cultivars from the *ex situ* collection in Srebrenik grouped in all three RPPs. Only RPP2 did not contain any of the international reference cultivars. The highest gene diversity (0.78), as well as average number of alleles (7.4) was detected within the RPP3 (Table 2). This is not surprising considering that this group had almost twice as many members as RPP1 and RPP2. Overall 24 alleles were unique for RPP3, while RPP1 and RPP2 had nine and 12 alleles respectively that were exclusive for each of the two mentioned reconstructed populations.

Table 3. Fst (WEIR and COCKERHAM, 1984; estimated with SPAGeDI 1.2) based on 10 SSR loci for the three reconstructed populations (RPPs) defined by Structure (PRITCHARD et al., 2000) (42 genotypes with qI>75%).

	RPP, K=3 (42 genotypes with *qI*>75%)	
Locus code	Fst	P-value
CH01H01	0.105	<0.0001
CH05E03	0.067	<0.0001
CH05E04	0.194	<0.0001
CH01H02	0.164	<0.0001
CH02C02a	0.077	<0.0001
CH04E02	0.054	0.029
CH01H10	0.063	0.003
CH02D08	0.063	<0.0001
CH02C02b	0.063	0.150
CH02C06	0.085	<0.0001
All loci	0.096	<0.0001

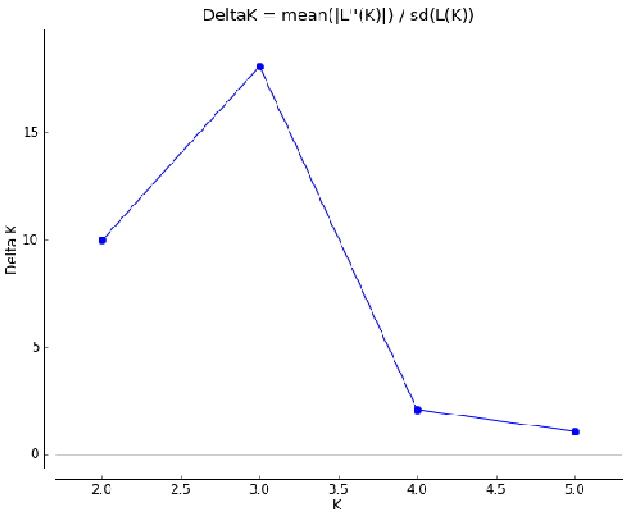


Fig. 1. Plot of delta *K* values from the Structure analyses of 51 apple accessions maintained at the ex situ collection in Srebrenik, obtained through Structure harvester ver. 0.6. application (EARL and VONHOLDT, 2011).

High and significant Fst value (0.096; P<0.0001) (Table 3) between the three RPPs indicates a substantial differentiation. Analyses of molecular variance detected that most of the variance was retained within the RRPs (91%), while 9% (P<0.001) was attributed to the differences among the analyzed RPPs of accessions (Table 4). The largest differentiation between RPPs was detected among RPP1 and RPP2 (18.8%) and the lowest among RPP2 and RPP3 (15.8%). The variance among all three RPPs (9%) was lower than the values reported by PEREIRA-LORENZO *et al*. (2008) (14.41%) for the three RPPs constructed with Spanish and international apple cultivars. However, even the lowest variance detected between individual pairs of RPPs in our study (15.8% for RPP2 and RRP3) is higher than the variance reported by GASI *et al*. (2013) (13%) for the two RPPs constructed with 108 apple accessions both from the *ex situ* collection and on farm locations. Also, the highest variance among individual pairs of RPPs, reported so far on apple germplasm (17%) (URRESTARAZU *et al*. 2012) is in fact lower than the variance obtained in this study among RPP1 and RPP2 (18.8%). Large and significant differentiation between individual RPPs detected through the analyses of molecular variance indicate that apple germplasm maintained at the *ex situ* collection in Srebrenik possess an interesting genetic structure. The specific structure reported here is possibly caused by the diverse origin of the examined apple accessions. The fact that two RPPs containing almost exclusively traditional apple accessions (RRP1 and RPP2), still differentiate significantly (18.8%; P<0.001) (Table 4) confirms that B&H apple germplasm is very heterogeneous. In order to visualize the genetic relationships between the three RRPs, a multivariate approach (FCA - factorial correspondence analysis) was used (Fig. 2). The distance between each of the three RRPs in relation to others is relatively equal, which is in concordance with the results of the AMOVA (Table 4). The differentiation between RPP1 and RPP2 is also evident in the FCA.

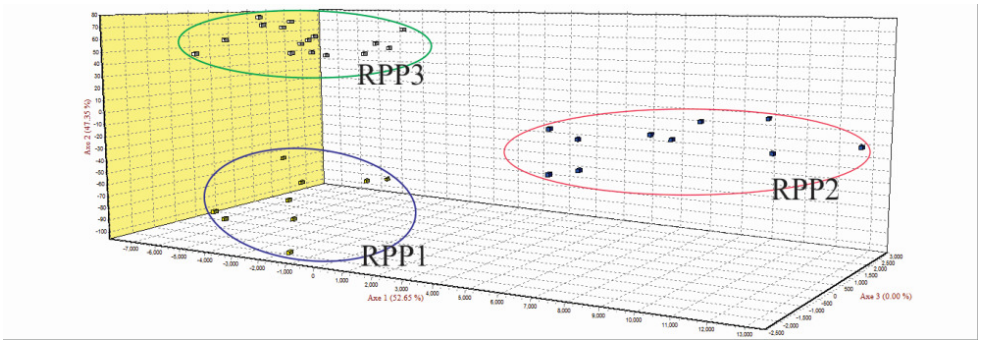


Fig. 2. Multivariate analysis (factorial correspondence analysis) of simple sequence repeat data for reconstructed populations (RPPs) calculated using Structure (PRITCHARD *et al*., 2000) (only diploid genotypes with *qI* greater than 75%).

Table 4. Analysis of molecular variance (AMOVA) based on the 10 SSR loci of all three RPPs reconstructed from apple accessions maintained at the ex situ collection Srebrenik by Structure (PRITCHARD et al., 2000); as well as for each of pair of the obtained RPPs.

Source of variation	df	Variance components	Total variance (%)	f_{CT}	P-value
All RPPs (42 accessions with *qI*>75%)					
Within groups:	39	65.1	91	0.09	<0.001
Among groups:	2	6.45	9		
Total	41	71.55			
RPP1 and RPP2 (N=22)					
Within groups:	20	63.11	81.2	0.18 8	<0.001
Among groups:	1	14.6	18.8		
Total	21	77.71			
RPP1 and RPP3 (N=31)					
Within groups:	29	65.99	84.1	0.15 9	<0.001
Among groups:	1	12.51	15.9		
Total	30	78.51			
RPP2 and RPP3 (N=31)					
Within groups:	29	65.58	84.2	0.15 8	<0.001
Among groups:	1	12.34	15.8		
Total	30				

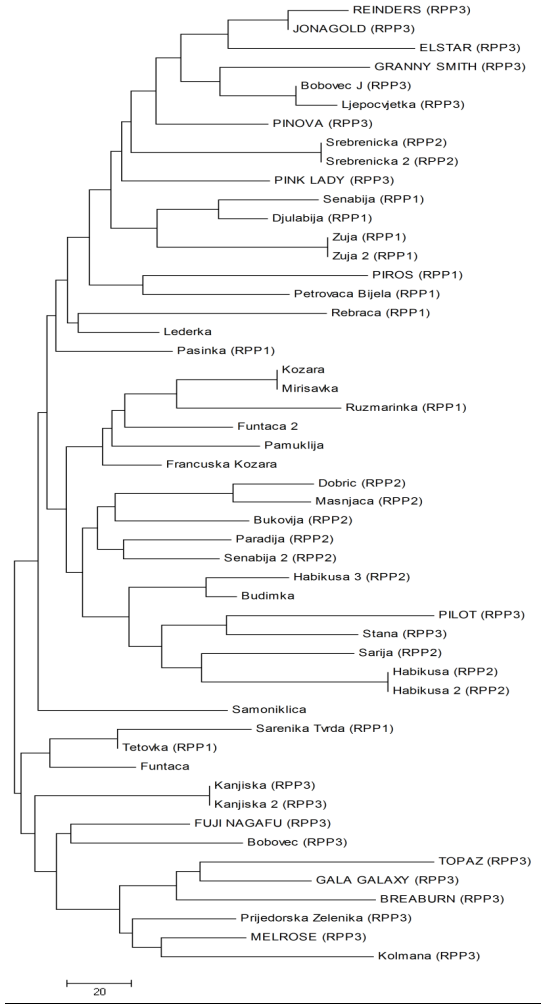
d.f. degree of freedom.

Genetic relationships

NJ cluster analysis, based on the Bruvo genetic distance (BRUVO *et al*., 2004), grouped all but one apple accessions (‘Samoniklica’) into three major clusters (Fig. 3). The first major cluster consists out of equal number of accessions from RPP1 and RPP3. Only one genotype from the RPP2 (‘Srebrenička’) wit *qI* greater than 75% grouped in this cluster. The second major cluster is dominated by accessions from RPP2, which is in fact the only group that does not contain any of the international reference cultivars. Similarly, the third major cluster belongs almost exclusively to accessions from RPP3, the group mainly consisting out of international reference cultivars. Out of 38 traditional B&H apple cultivars, analyzed in our study, ‘Ljepocvjetka’, ‘Bobovec’ and ‘Bobovec J’ grouped closest to the international reference cultivars. Considering that ‘Ljepocvjetka’ is a synonym of ‘Bellflower’ and that ‘Bobovec’ is a synonym of ‘Bohnapfel’, a cultivar introduced from the Rhineland, obtained results of the cluster analyses are not surprising. Current hypothesis, based on the results of earlier molecular studies done on apple germplasm in B&H (GASI *et al*., 2010; GASI *et al*., 2013), proposes that apple accessions which are more closely related to international, commercial cultivars were in fact

introduced during the reign of Austrian–Hungary Empire over Bosnia and Herzegovina. On the other hand, accessions which clearly differentiate from the international ones, have presumably been introduced to B&H at a much earlier stage, during the reign of the Ottoman Empire.

Fig. 3. Neighbor joining cluster analysis based on polymorphisms of 10 simple sequence repeat loci in 38 traditional B&H apple cultivars and 13 reference apple cultivars (written in capital letters) using Bruvo genetic distance (BRUVO et al., 2004). In brackets are the reconstructed populations (RPP1, RPP2 or RPP3) to which each cultivar is assigned with probability of membership qI greater than 75%.



The fact that 'Bobovec J' is not closely related to 'Bobovec' indicates a misidentification of this apple accession. Several other misidentifications were also detected through the NJ cluster analysis. For instance, in spite of the name under which it is registered, the accession 'Francuska kožara' ('Reinette grise francaise') did not cluster closely to the international cultivars indicating a previous misidentification based on the pomological data. One synonym was detected ('Kožara' had the identical SSR profile as 'Mirisavka'). Three homonyms were also revealed: 'Senabija' and 'Senabija 2' are in fact not even closely related; 'Funtača' and 'Funtača 2' were present in different major clusters and therefore not identical or closely related; 'Habikuša 3, although in the same major cluster as the other two accessions registered as 'Habikuša' was not genetically identical to them. The misidentification of the accession registered under the name of 'Funatača 2' was understandable because this genotype had very large fruits, as is the case with the original 'Funatača' accession. Other three cases of misidentification are probably due to unreliability of using pomological data for identification of traditional apple cultivars. However, four genotypes ('Srebrenička 2', Žuja 2', 'Habikuša 2' and 'Kanjiška 2') were correctly identified as synonyms of the original accessions ('Srebrenička', Žuja', 'Habikuša' and 'Kanjiška'). Accuracy of the phenotypic identification in these cases could be due to some specific pomological traits that these cultivars possess which distinguishes them from the other apple accessions in the collection.

CONCLUSION

Results of this study allowed us to identify all homonyms and redundancies within the Srebrenik collection. Cluster analyses revealed that cultivars such as 'Bellflower' and 'Bohnapfel', introduced more recently to Bosnia and Herzegovina, grouped closely with international reference cultivars. On the other hand, traditional accessions maintained in Srebrenik, which were presumably introduced during the reign of the Ottoman Empire, differentiated quite clearly from reference genotypes. Results of the structure analyses indicate a heterogeneous genetic structure of the analyzed accessions, which could be useful for future breeding programs, especially for widening the genetic base of commercial apple cultivars.

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GENETSKA STRUKTURA AKSEŠNA JABUKE ODRŽAVANIH *EX SITU* U BOSNI I HERZEGOVINI ISPITANA MIKROSATELITSKIM MARKERIMA

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Izvod

U cilju sticanja uvida u genetsku strukturu germplazme jabuke održavane *ex situ* u Srebreniku, 14 aksešna iz date kolekcije genotipizirano je korišćenjem 10 SSR markera. Dobijeni SSR profili dodati su već postojećoj bazi podataka sačinjenoj od prethodno karakteriziranih 24 tradicionalnih i 13 internacionalnih, referentnih sorti koji se održavaju u istoj kolekciji. Bayesian analiza sprovedena u STUCTURE računarskom programu grupisala je 42 od 51 analiziranog aksešna jabuke (38 tradicionalnih i 13 internacionalnih) u tri RPP-a (rekonstruisane panmiktičke populacije) sa verovatnoćom članstva *ql* veće od 75%. Gotovo sve internacionalne, referentne sorte grupisale su se u RPP3, dok se tradicionalne BiH sorte iz Srebrenika mogu naći u sva tri RPP-a. Visoka i statistički značajna diferencijacija uočena je između pojedinačnih RPP-ova i dodatno potvrđena sa FCA (faktorska korespondentna analiza). NJ klaster analiza, bazirana na Bruvo genetskoj udaljenosti, otkrila je da od 38 ispitivanih tradicionalnih BiH sorti jabuke, 'Ljepocvjetka', 'Bobovec' i 'Bobovec J.' se grupišu najbliže internacionalnim, referentnim sortama. Dostupni podaci ukazuju da za razliku od velikog broja BiH sorti jabuke koje su introdukovane tokom vladavine Osmanskog carstva, 'Ljepocvjetka' i 'Bobovec' su verovatno introdukovane na ove prostore nešto kasnije. Klaster analiza je takođe omogućila detekciju jednog sinonima i tri homonima unutar kolekcije. U četiri slučaja, prethodno sprovedena identifikacija na osnovu fenotipa je potvrđena genetskim analizama. Rezultati analize genetske strukture ukazuju na heterogenu strukturu analiziranih aksešna. Ova karakteristika BiH germplazme jabuke može biti interesantna za buduće oplemenjivačke programe.

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