HORTSCIENCE 48(1):13–21. 2013.

Evaluation of Apple (*Malus* ×*domestica*) Genetic Resources in Bosnia and Herzegovina Using Microsatellite Markers

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Additional index words. local cultivars, genetic differentiation, genetic structure

***Abstract*. There is a need for classifying and conserving local apple cultivars from two main regions in Bosnia and Herzegovina (B&H). Consequently, 71 local apple accessions (31 from Sarajevo and 40 from eastern Bosnia) were evaluated with a set of 10 simple sequence repeats (SSRs). These accessions were compared with 37 reference cultivars (24 traditional B&H and 13 international cultivars maintained at the ex situ collection Srebrenik) to determine synonyms, homonyms, and possible introgression of foreign genotypes into the local apple germplasm. Using 10 primer pairs of microsatellites, we were able to amplify 135 alleles for the 71 local apple accessions. Detection of more than two different alleles per locus was observed for 34 accessions. Fourteen different homonyms and 12 synonyms were identified among all the apple cultivars analyzed. Analysis of molecular variance (AMOVA) revealed a significant genetic differentiation between most of the groups analyzed but not between accessions from Sarajevo and eastern Bosnia. Bayesian method and admixture analysis of the allele frequency allowed classification of all accessions analyzed and found that they fell into two main groups [reconstructed panmictic populations (RPPs)]. Strong genetic differentiation between these two groups was detected using AMOVA ($f_{CT} = 0.130$; $P < 0.001$). Analysis of the genetic structure indicates that overall, approximately half of the local apple cultivars from Sarajevo and eastern Bosnia (52% and 45%, respectively) grouped in the RPP1 consisting mainly out of international reference cultivars, whereas the other half grouped in the RPP2 with traditional B&H reference cultivars. Both neighbor joining (NJ) cluster analysis based on Bruvo genetic distance and factorial correspondence analysis (FCA) confirmed the results of the genetic structure analysis. The molecular data show that both apple accessions from Sarajevo and from eastern Bosnia represent an interesting source of diversity, which needs to be conserved.**

Traditional apple cultivars in B&H have significantly higher genetic and morphological diversity than modern international cultivars grown in this country (Gasi et al., 2010, 2011). Because genetically heterogeneous material represents a potential source of positive pomologic traits and resistance to biotic and abiotic stress (Kellerhals et al., 2004), local traditional B&H apple cultivars may be useful for inclusion in international apple breeding programs and therefore must be conserved. To conserve the aforementioned genetic resource, the main ex situ collection in the country was established in the fruit tree nursery Srebrenik (Spionica, B&H, lat. 44°45′45″N, long. 18°29′49″E) in 2000. Aside from local traditional B&H cultivars, the established collection maintains many international cultivars widely grown in the region, which often serve as reference genotypes in apple germplasm studies. Genetic assessment of 39 accessions, 24 well-known traditional B&H cultivars and 15 modern international cultivars, maintained at the ex situ collection Srebrenik has recently been performed using molecular markers (Gasi et al., 2010). The molecular analyses revealed a clear differentiation between traditional B&H cultivars and some of the most widely cultivated modern cultivars in the region, confirming the importance of conserving B&H apple germplasm. Unfortunately, there are high costs associated with maintaining a large ex situ collection and in a post-conflict country with limited resources, there is an even greater need for increased efficiency and elimination of redundancies by avoiding duplication and mislabeling.

In 2010 and 2011, an effort was made to fill the gaps in the apple collection Srebrenik. During the original collecting missions, completed in 2000, which resulted in establishment of the collection, two geographical areas of Bosnia and Herzegovina were omitted: the capitol Sarajevo with the surrounding area and large parts of eastern Bosnia. The mentioned areas had at that time been significantly depopulated as a result of the war and only in recent years have most of the original inhabitants returned to their homesteads. To ensure that the existing ex situ apple collection contains as much as possible of the diverse B&H apple germplasm, we surveyed the Sarajevo region and parts of eastern Bosnia for local apple cultivars. This was done with the help of local agricultural experts and the traditional knowledge gathered from the farmers living in surveyed parts of the country. The results of the survey indicate a presence of a large number of local cultivars that are grown in the aforementioned regions. However, without knowing the genetic identity of these genotypes, it is impossible to estimate if local cultivars from the surveyed areas can be considered part of traditional B&H apple germplasm or if they are mostly mislabeled international cultivars. Because DNA markers are much more efficient in revealing mislabeled plant accession compared with traditional pomological characterizations (Nybom and Weising, 2010), we chose this approach in our study. SSR molecular markers have shown great promise as tools for managing *Malus* ex situ germplasm collections as well as for collection and preservation strategies of these genetic resources (Hokansson et al., 1998). Examining ex situ collections with microsatellite markers has so far been performed in a number of studies (Garkava-Gustavsson et al., 2008; Gharghani et al., 2009; Guarino et al., 2006; Guilford et al., 1997; Hokanson et al., 2001; Pereira-Lorenzo et al., 2006; Ramos-Cabrer et al., 2007; Urrestarazu et al., 2012; Van Treuren et al., 2010), which has greatly contributed to more efficient management and use of the analyzed collections. Aside from managing established collection, SSR markers have also proved very useful in determining synonyms and genetic relationships of local apple cultivars found on farms (Bassil et al., 2009; Pereira-Lorenzo et al., 2008; Routson et al., 2009).

The objectives of this study therefore were: 1) to compare genetic profiles of local apple cultivars from Sarajevo and eastern Bosnia with a database constructed for previously characterized traditional and international apple cultivars in B&H using a set of 10 SSR markers; and 2) to evaluate the need for conservation of local apple cultivars from Sarajevo and eastern Bosnia.

Materials and Methods

A total of 71 accessions were sampled from a number of backyards and small farms in Sarajevo and eastern Bosnia (31 from Sarajevo and 40 from eastern Bosnia) (Table 1). The apple accessions, all grafted on generative rootstocks, are maintained in good condition by farmers and their fruits are used for a wide range of purposes (fresh consumption, manufacturing of traditional desserts, and production of apple brandy). Farmers identified each accession as a local, traditional B&H cultivar. In addition, 37 apple accessions (24 well-known traditional B&H cultivars and 13 international cultivars widely grown in the region) maintained at the ex situ collection Srebrenik and genotyped in a previous study by Gasi et al. (2010) using the same set of SSR markers were included as reference cultivars (Table 2). Traditional B&H genotypes used as reference cultivars in this study are part of the apple germplasm introduced in Bosnia and Herzegovina during the reign of the Ottoman and later Austrian–Hungary Empire (Bubić, 1952). The mentioned germplasm has extensively been selected by farmers but has yet to be included in plant breeding programs established in the region.

Simple sequence repeat analyses. Tissue samples (leaves) for DNA analyses were collected in the spring of 2011 immediately after bloom from a single tree for each accession

Table 1. Seventy one apple accessions (31 sampled from farms in Sarajevo and 40 from farms in eastern Bosnia) analyzed in this study using 10 simple sequence repeat markers.[z]

Local name	Accession number	Original place of registration
Sarajevo accessions		
Crvenika	SG50	Škaljića sokak
Cvjetača	SG51	Mošćanica
Mađarica	SG52	Mošćanica
Lederka	SG53	Škaljića sokak
Šarenika	SG54	Širokača
Petrovača	SG55	Obhodža
Bobovača	SG56	Mošćanica
Žuta	SG58	Brusulje
Crvenka	SG59	Hrid-Jarčedoli
Zečije uši	SG60	Hrid-Jarčedoli
Cvjetača	J1	Širokača
Crvena lederica	J3	Širokača
Siva lederica	J4	Širokača
Senabija	J5	Širokača
Šarenika kasna	J8	Širokača
Šupa	J12	Obhodža
Cvjetača	J13	Obhodža
Kanada	J15	Obhodža
Kolarača crvena	J16	Obhodža
Budimka	J17	Obhodža
Mirisavka	J18	Obhodža
Petrovača	J21	Škaljića sokak
Bistrica	SS1	Škaljića sokak
Dobrinjka	SS2	Škaljića sokak
Lederica	SS3	Baruthana 13
Limun	SS4	Škaljića sokak
Ljepocvjetka	SS5	Brusulje
Šarenika	SS6	Škaljića sokak
Senabija	SS7	Baruthana 13
Šarenika slatka	SS8	Širokača
Srebrenka	SS9	Škaljića sokak
Eastern Bosnia accessions		
Cvjetača	GC04	Zupčići
Dobrinjka	GD08	Zupčići
Lederica	GL02	Zupčići
Senabija	GS01	Zupčići
Srebrnjača	GS06	Zupčići
Sulija	GS07	Zupčići
Zelenika	GZ03	Zupčići
Zečija glava	GZ05	Zupčići
Ananas	GA11	Ilovača
Amerikanka	GA40	Zupčići
Bistrica	GB10	Bistrica
Butulija	GB14	Vranići-Zabašča
Bedrika	GB16	Vranići-Zabašča
Bihorka	GB27	Vranići-Kupušnjak
Baščenka	GB29	Kodžaga_polje
Babovača	GB30	Zupčići
Bjelka	GB44	Vranići-Kupušnjak
Bijela ruzmarinka	GB45	Foča
Carkonstatin	GC15	Kostenik-Klupca
Crvenka	GC35	Osanica
Dobrinjkina majka	GD38	Osanica
Kanada	GK13	Zupčići
Lorinka	GL31	Kazagići
Limunka	GL36	Vranići
Ljutika	GLJ34	Kruščica
Muslimača	GM18	Kostenik-Klupca
Misirka	GM26	Ilovača
Ovčiji_nos	GO42	Zupčići
Petrovača žuta	GP09	Zupčići
Pazarka	GP17	Kostenik-Klupca
Prisatka	GP20	Kostenik-Klupca
Srčika	GP22	Ilovača
Posavka	GP23	Mašići
Petrovača crvena	GP43	Zupčići
Rebrača	GR19	Kostenik-Klupca
Ranka	GR33	Vran Potok
Šarenika	GS24	Kostenik-Klupca
Samoniklica	GS37	Vranići
Švabska zelenika	GS41	Zupčići
Šimširka	GS46	Kostenik-Klupca

[z]Collection information includes local name, accession number, and original place of registration.

Received for publication 1 Oct. 2012. Accepted for publication 5 Nov. 2012.

This study was funded by the Norwegian government through HERD (Programme for Higher Education, Research and Development) project "Evaluation of fruit genetic resources in Bosnia-Herzegovina with the aim of sustainable, commercial utilization" ref. no. 332160 UE.

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Table 2. Twenty-four previously genotyped traditional Bosnia and Herzegovina (B&H) and 13 international apple accessions maintained in the ex situ collection Srebrenik used as reference cultivars in this study.

	Traditional B&H reference cultivars	Accession no.[z]		International reference cultivars	Accession no.
1	Paradija	FAS 344	1	Pink Lady	FAS 380
2	Šarenika tvrda	FAS 345	2	Topaz	FAS 381
3	Sarija	FAS 346	3	Fuji Nagafu	FAS 382
4	Bobovec Jon	FAS 347	4	Golden Reinders	FAS 383
5	Dobrić	FAS 348	5	Gala Galaxy	FAS 385
6	Ruzmarinka	FAS 349	6	Pinova	FAS 386
7	Lijepocvjetka	FAS 350	7	Pilot	FAS 387
8	Lederka	FAS 351	8	Piros	FAS 389
9	Budimka	FAS 352	9	Braeburn	FAS 390
10	Senabija	FAS 353	10	Melrose	FAS 391
11	Rebrača	FAS 354	11	Elstar	FAS 392
12	Samoniklica	FAS 355	12	Granny Smith	FAS 393
13	Žuja	FAS 356	13	Jonagold	FAS 394
14	Bobovec	FAS 357			
15	Petrovača bijela	FAS 358			
16	Tetovka	FAS 359			
17	Prijedorska zelenika	FAS 360			
18	Pamuklija	FAS 361			
19	Bukovija	FAS 362			
20	Habikuša	FAS 363			
21	Kanjiška	FAS 364			
22	Srebrenička	FAS 365			
23	Funtača	FAS 366			
24	Đulabija	FAS 367			

[z] International accession number according to EURISCO.

located in Sarajevo and eastern Bosnia. DNA extraction was performed with a Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) according to the protocol included in the kit. Ten primer pairs used for SSR amplifications, previously published by Gianfranceschi et al. (1998) and Liebhard et al. (2002), were chosen in cooperation with a research team from Wageningen University and Research Center and Central gene bank of Netherlands. Polymerase chain reaction (PCR) amplification of SSR sequences was performed in a Veriti TM Thermal Cycler (Applied Biosystems, Foster City, CA) using fluorescent-labeled primers, which enabled the detection of PCR products using an ABI 3130 Genetic Analyzer (Applied Biosystems). All PCR amplifications were performed as described in Gianfranceschi et al. (1998). The PCR product was diluted with ddH20 (1:50), then added to 8.75 µl HiDi and 0.25 µL Genescan 500 LIZ size standard. Data were analyzed using the software package GeneMapper 4.0 (Applied Biosystems).

Biostatistical analyses. SSR profiles obtained by genotyping 71 accessions from Sarajevo and eastern Bosnia were added to the existing microsatellite database constructed in a previous study on apple accessions maintained at the main ex situ apple collection in B&H. All accessions analyzed were divided into four groups for biostatistical purposes: Sarajevo accessions, eastern Bosnia accessions, international reference cultivars, and traditional B&H reference cultivars.

Population genetics software SPAGeDI 1.2 (Hardy and Vekemans, 2002) was used for calculating allele frequencies, gene diversity (Nei, 1978), and F statistics (Weir and Cockerham, 1984). Analyses of molecular variance (Exoffier et al., 1992), based on the stepwise mutation model (Ohta and 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) and Shannon-Weaver's information index was calculated using GenoDive software. Both programs are part of the GenoType/GenoDive package (Meirmans and Van Tienderen, 2004).

Neighbor-joining cluster analysis, based on the mentioned genetic distance, was performed in MEGA 5 software (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011). A multivariate analyses, FCA, based on allele frequencies was performed using Genetix 4.02 (Belkhir et al., 2001), which meant excluding the triploid genotypes, as proposed by Pereira-Lorenzo et al. (2008).

To examine population structure and assign individual genotypes to either traditional B&H or international reference cultivars apple germplasm, we used the Bayesian model-based cluster procedure within Structure Version 2.2.3 (Pritchard et al., 2000), which is suitable for analysis of diploids and polyploids. We computed K (unknown) RPPs of individuals testing K (log-likelihood) = 1 to 5 for 108 accessions assuming that sampled cultivars were from unknown origin anonymous trees. Assignment of one cultivar in a RPP was provided by a probability of membership *qI* chosen at 80% according to similar studies (Breton et al., 2008; Pereira-Lorenzo et al., 2008; Urrestarazu et al., 2012). Tests were done based on an admixture model in which the allelic frequencies were correlated and a burn-in period of 200,000 and 500,000 iterations for data collection was applied (Urrestarazu et al., 2012).

Results and Discussion

Simple sequence repeat polymorphism. Of 71 accessions from Sarajevo and eastern Bosnia, 64 showed a unique SSR profile (Table 3). When the reference cultivars, genotyped in a previous study (24 well-known traditional B&H cultivars and 13 international cultivars widely grown in the region), were included, 14 different homonyms and 12 synonyms were detected among the 108 accessions. Five accessions from eastern Bosnia and three from Sarajevo were already present in the Srebrenik collection under different names. Two accessions registered as local cultivars in the Sarajevo region had in fact the same SSR profile as the international reference cultivar Jonagold, indicating the presence of international genotypes in Sarajevo local apple germplasm. After excluding synonyms and duplicates, 96 different genotypes remained in the new database.

Detection of more than two different alleles per locus was observed for 34 accessions (18 accessions from Sarajevo and 16 from Eastern Bosnia), or 48% of 71 analyzed local cultivars. Seven of the mentioned accessions had only one locus with third allele, whereas most others had several SSR loci with a third allele. Although flow cytometry is most commonly applied for identifying triploids, because none of the used primer pairs displayed the ability to amplify more than one locus in our study, a presence of a third allele indicates a triploid state. A detected percentage of triploids is considerably higher than that reported for the local Spanish apple cultivars (29%) (Ramos-Cabrer et al., 2007), as well as compared with the data from the previous molecular study on B&H apple germplasm (21%) (Gasi et al., 2010).

Ten primer pairs of microsatellites managed to amplify 135 alleles or an average of 13.5 alleles per locus for the 71 apple accessions sampled from Sarajevo and eastern Bosnia, which represents a 30% increase compared with the results of the previous study on apple germplasm in Bosnia and Herzegovina (10.4 alleles per loci) (Gasi et al., 2010). Number of detected alleles per locus ranged from seven alleles for CH01H02 to 22 alleles for CH02C02a (Table 4). Average number of alleles per locus obtained in this study was also higher than that reported for the Italian, Danish, and Swedish material as well as foreign and local cultivars from northwestern Spain (Garkava-Gustavsson et al., 2008; Guarino et al., 2006; Larsen et al., 2006; Pereira-Lorenzo et al., 2006). Of the two investigated B&H regions, a higher number of alleles was amplified among apple accessions from eastern Bosnia (12.4) compared with Sarajevo accessions (10.5). Lowest numbers were detected for international reference cultivars (6.3).

Analyses of the allele frequency for all the loci revealed SSR alleles specific for each of the four groups of accessions examined. A total of 23 alleles was exclusively present

Table 3. Simple sequence repeat (SSR) profiles (allele sizes expressed in base pairs) of 71 apple accessions from Bosnia and Herzegovina (31 sampled from farms in Sarajevo and 40 from farms in eastern Bosnia) analyzed using 10 SSR markers.

Apple accessions	Accession no.	CH01H01	CH05E03	CH05E04	CH01H02	CH02C02a	CH04E02	CH01H10	CH02D08	CH02C02b	CH02C06
Sarajevo accessions											
Crvenika	SG50	115:131	159:180	160:168	235:247	174:176	150:158	095:102	229:254	109:109	236:253
Cvjetača	SG51	117:131	163:180	153:160	245:247	156:178	154:158	102:113	223:246	109:109	236:253
Mađarica	SG52	113:117:119	159:159	160:168	235:239:243	156:180	145:158	102:106:113	208:212	109:109	253:253
Lederka	SG53	113:117:131	163:167	149:160	235:247	130:182	139:158	095:102	212:229:254	109:113	230:251:253
Šarenika	SG54	115:117:131	159:187	160:174:176	235:239	171:178	143:150:154	095:102	225:254	109:109	253:253
Petrovača	SG55	113:117	159:172	162:162	235:235	167:167	154:158	106:106	212:254	075:113	232:232
Bobovača	SG56	115:119:131	163:170:174	160:164	235:247	130:167:186	154:158:166	095:102	225:229:254	075:109:113	230:241:253
Žuta	SG58	113:117:119	161:161	160:168	235:235	167:184	158:158	102:102	212:225:254	109:113	234:241:249
Crvenka	SG59	113:115	169:169	160:162	235:243	184:188	154:158	102:106	216:218	109:113	255:255
Zečije uši	SG60	117:119	157:178	160:172	245:247:249	167:180	158:158	102:102	212:254	109:109	251:255:257
Cvjetača	J1	117:131	163:180	153:160	245:247	156:178	154:158	102:113	223:246	109:109	236:253
Crvena lederica	J3	105:127:131	159:174:193	160:168	243:247	167:176:180	154:154	095:102	214:225:229	109:119	230:241:253
Siva lederica	J4	105:127:131	159:174:193	160:168	243:247	167:176:180	154:154	095:102	214:225:229	109:119	230:241:253
Senabija	J5	117:119:131	163:178:184	160:168	247:249	176:180:186	150:158	095:113	223:225:229	109:113	236:241:253
Šarenika kasna	J8	115:131	172:191	160:160	235:243	169:184	158:166	102:102	212:214	119:119	253:257
Šupa	J12	111:117:119	161:187	162:168:172	235:243:249	156:167:176	158:158	100:104	212:216:223	109:113	247:255:259
Cvjetača	J13	117:131	163:184:189	160:168	235:245	130:171:178	143:154:158	95:106	212:223:254	109:113	236:253
Kanada	J15	117:131	163:184:189	160:168	235:245	130:171:178	143:154:158	95:106	212:223:254	109:113	236:253
Kolarača crvena	J16	113:117:119	163:163	158:168	235:243	156:180	145:158	102:106:113	208:212	119:119	234:253
Budimka	J17	111:111	161:191	160:172	235:249	176:180	158:158	102:104	216:223	109:109	234:247
Mirisavka	J18	115:131	172:191	160:160	235:243	169:184	158:166	102:102	212:214	109:109	253:257
Petrovača	J21	111:121	159:191	160:160	235:247	169:169	154:158	106:117	210:212	109:113	230:234
Bistrica	SS1	117:119	165:165	160:168	235:235	169:169	158:158	100:102	212:225	113:113	234:249
Dobrinjka	SS2	113:129	161:161	160:162	235:247	130:186	152:158	095:102	229:254	109:109	241:253
Lederica	SS3	105:127:131	159:163:178	160:168	243:247	169:178:182	152:154	095:102	214:225:229	109:109	230:241
Limun	SS4	113:125	161:161	160:168	235:245	130:173	158:158	100:102	223:254	113:113	253:263:265
Ljepocvjetka	SS5	117:119:131	163:178:184	160:168	247:249	176:180:186	150:158	095:113	223:225:229	109:113	236:241:253
Šarenika	SS6	115:131	159:180	160:168	235:247	174:176	150:158	095:102	229:254	109:109	236:253
Senabija	SS7	115:117	172:178:191	160:162	235:249	134:171:182	143:158	102:104:106	214:216:218	109:113	234:257:259
Šarenika slatka	SS8	121:121	172:189	160:160	243:245	176:182	158:166	102:102	212:223	113:113	232:236
Srebrenka	SS9	111:115	193:193	160:172	245:249	167:178	158:158	102:107	212:229	113:113	218:253:259
Eastern Bosnia accessions											
Cvjetača	GC04	117:131	167:184	153:160	245:247	160:180	154:158	102:113	223:246	109:113	236:253
Dobrinjka	GD08	113:131	163:172	149:164	235:245	176:176	154:158	102:113	212:225	109:113	253:253
Lederica	GL02	113:131	163:182	153:160:168	235:247	130:180	150:158	102:107:113	254:254	109:113	245:253:261
Senabija	GS01	111:117	161:191	158:188	235:247	178:182	158:158	95:102	216:223	109:113:121	234:247
Srebrnjača	GS06	111:115	159:193	160:172	245:249	167:178	158:158	102:107	212:229	113:113	232:236
Sulija	GS07	113:117	172:172	160:168	235:245	178:188	130:154	102:113	212:223	109:109	236:257
Zelenika	GZ03	113:117:119	163:163	160:160	235:243	160:182:184	145:158	102:113	208:212	109:113	251:253
Zečija glava	GZ05	117:119	159:180	160:172	245:247:249	169:182	158:158	102:107	212:254	109:119	251:257
Ananas	GA11	117:121	161:161	149:160:168	235:247	144:180	158:158	95:102	229:229	109:113	245:253
Amerikanka	GA40	117:117	189:191	168:168	235:245	144:146	154:154	95:102	212:218	109:113	218:251
Bistrica	GB10	109:113	163:163	168:168	235:235	130:182	152:158	95:106	212:229	113:113	241:251
Butulija	GB14	117:119	172:172	162:168	235:243	134:182	158:158	102:106	214:254	109:113	236:259
Bedrika	GB16	105:127:131	163:178:197	160:168	243:247	169:178:182	152:154	95:100	214:225:229	109:113	230:241:253
Bihorka	GB27	117:119	159:159	160:172	235:249	146:182	156:158	91:102	212:218	109:113	257:263
Baščenka	GB29	117:119	163:191	158:160	235:235	169:176	158:166	102:102	218:225	113:117	234:253
Babovača	GB30	111:115	159:187	160:172	243:249	167:178	158:158	102:102	212:223	113:119	236:255

(*Continued on next page*)

Table 3. (*Continued*) Simple sequence repeat (SSR) profiles (allele sizes expressed in base pairs) of 71 apple accessions from Bosnia and Herzegovina (31 sampled from farms in Sarajevo and 40 from farms in eastern Bosnia) analyzed using 10 SSR markers.

Apple accessions	Accession no.	CH01H01	CH05E03	CH05E04	CH01H02	CH02C02a	CH04E02	CH01H10	CH02D08	CH02C02b	CH02C06
Bjelka	GB44	119:131	172:191	168:172	243:243	167:182	158:158	102:106	212:254	109:121	241:253:257
Bijela ruzmarinka	GB45	117:121	161:161	168:168	235:247	176:180	158:158	95:102	229:229	109:113	245:253
Car konstatin	GC15	105:111	163:180	153:160:162	235:247	180:184:186	154:154	95:102	212:246:248	109:113	218:255
Crvenka	GC35	121:131	167:174	160:193	235:243	146:182	150:152	95:106	214:252	113:117	230:255
Dobrinjkina majka	GD38	113:131	161:182	153:160:168	235:247	130:180	150:158	95:102	206:254	109:113	245:253:261
Kanada	GK13	113:117:131	163:167	162:162	235:247	130:182	139:158	95:102	212:229	109:113	230:251:253
Lorinka	GL31	115:119:131	167:172:178	160:164	235:247	169:188	154:158:166	95:102	225:229:254	109:113	230:241:253
Limunka	GL36	111:115	159:193	160:172	245:249	167:178	158:158	102:107	212:229	113:113	232:236
Ljutika	GLJ34	115:117:127	157:180:189	160:160	235:235	160:188	154:158	95:102	225:254	113:117	218:253
Muslimača	GM18	111:115	161:161	160:176	235:235	146:169	158:158	102:102	212:212	113:123	245:247
Misirka	GM26	115:115	172:180	160:162	235:235	182:203	154:158	102:104	214:218	109:113	253:257
Ovčiji nos	GO42	113:117:119	159:180	191:193	245:247:249	169:182	158:158	102:102	212:254	109:113	251:257
Petrovača žuta	GP09	113:115	163:172	170:170	235:235	146:180	150:150	102:106	216:225	109:109	218:253
Pazarka	GP17	113:117	172:172	162:162	235:245	160:171	156:158	104:106	212:214	109:115	245:251:257
Prisatka	GP20	113:131	161:161	160:168	243:247	134:180	150:158	95:102	225:225	109:113	247:251
Srčika	GP22	121:123	163:189	153:160	231:247	146:182	154:158	95:95	212:254	109:113	232:253
Posavka	GP23	115:121	163:163	158:176	235:249	203:238	150:158	106:113	212:218	109:113	245:253
Petrovača crvena	GP43	115:117:131	161:189	160:174:176	235:239	173:180:203	143:150:154	95:102	225:254	109:113	218:253
Rebrača	GR19	117:119	189:189	153:160	239:247	132:158	158:158	95:102	225:246	109:113	255:265
Ranka	GR33	115:131	163:193	160:172	235:245	167:178	130:158	95:102	218:229	109:113	232:253
Šarenika	GS24	111:143	159:161	172:172	235:249	167:182	158:158	102:102	212:216	119:119	236:245
Samoniklica	GS37	113:121	189:191	176:186	235:249	176:178	130:166	106:113	212:216	109:113	253:257
Švabska zelenika	GS41	113:117:119	163:163	160:168	235:243	160:182:184	145:158	102:106	208:212	109:113	251:253
Šimširka	GS46	111:131	191:191	174:174	243:245	167:186	158:166	102:102	218:229	113:117	236:253

among the accessions from eastern Bosnia, whereas nine alleles were only detected among accessions registered in Sarajevo. Nine different alleles were specific for traditional B&H reference cultivars, whereas only three alleles were specific for the international reference cultivars.

Gene diversity for individual markers ranged from 0.62 for CH02C02b to 0.93 for CH02C02a. Average gene diversity estimated in this study was 0.80 (0.79 for Sarajevo and 0.80 for eastern Bosnia accessions), higher than the values published by Hokanson et al. (2001) (0.62), Liebhard et al. (2002) (0.74), and Guarino et al. (2006) (0.73). Gene diversity for 71 accessions from Sarajevo and eastern Bosnia was identical to the one calculated for 109 traditional Spanish cultivars (0.80) (Pereira-Lorenzo et al., 2006) and slightly lower than the values reported by Urrestarazu et al. (2012) (0.82) who analyzed 538 apple cultivars in northeastern Spain. Shannon-Weaver's information index (*I*) showed diversity to be highest in eastern Bosnia accessions ($I = 1.59$) and the lowest in international reference cultivars ($I = 1.11$). Traditional B&H reference cultivars and accessions from Sarajevo had similar values for this index (I = 1.38 and I = 1.39, respectively). It is important to note that this measure is suitable only when comparing groups of similar size (similar number of analyzed genotypes). Based on the number of amplified alleles, specific alleles, and gene diversity, accessions from eastern Bosnia seem to represent the most genetically diverse group of all four analyzed.

Values for the inbreeding coefficient Fit, calculated for the 71 apple accessions sampled from Sarajevo and eastern Bosnia, ranged from –0.159 for CH01H10 to 0.182 for CH05E03 with an average value of –0.047 for all loci (Table 5). The average Fit value calculated for all 108 analyzed accessions (international and traditional B&H reference cultivars included) was also very low (–0.009). Both values were much lower than those previous published for Bosnian, Spanish, and Iranian apple accessions (Gasi et al., 2010; Gharghani et al., 2009; Pereira-Lorenzo et al., 2008). Because high Fit values indicate heterozygosity deficiency, a lower Fit value obtained in this study points to no loss of heterozygosity among the analyzed accessions. Very low values obtained for Fis coefficient (–0.050 for Sarajevo and eastern Bosnia accessions and –0.028 for all four groups of accessions) is in concordance with the calculated Fit inbreeding coefficient.

The differentiation between apple accessions from Sarajevo and eastern Bosnia (Fst value) (Weir and Cockerham, 1984) for all 10 loci was very low (0.002; *P* value nonsignificant), possibly indicating a common genetic structure of the genotypes sampled from these two regions. Fst value calculated for all 108 analyzed accessions (international and traditional B&H reference cultivars included) was significant (0.019; $P < 0.0001$) but much lower than that reported by the

Table 4. Allele size range (bp) for all the analyzed apple accessions, number of alleles per locus, and gene diversity (Nei, 1978) based on 10 simple sequence repeat loci for 31 apple accessions from Sarajevo, 40 apple accessions from eastern Bosnia, 71 accession genotyped in this study, 24 traditional Bosnia and Herzegovina (B&H), and 13 international reference apple cultivars from ex situ collection Srebrenik.

		Sarajevo accessions (N = 31)		Eastern Bosnia accessions (N = 40)		Sarajevo and eastern Bosnia accessions (N = 71)		Traditional B&H reference cultivars (N = 24)		International reference cultivars (N = 13)	
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	No. of alleles	Gene diversity
CH01H01	105/145	11	0.87	12	0.87	14	0.87	9	0.87	6	0.84
CH05E03	153/197	17	0.92	16	0.90	19	0.91	12	0.86	8	0.88
CH05E04	149/193	10	0.68	15	0.84	15	0.78	8	0.81	7	0.76
CH01H02	231/251	6	0.77	7	0.75	7	0.76	7	0.67	6	0.75
CH02C02a	130/238	15	0.93	20	0.93	22	0.93	15	0.91	10	0.87
CH04E02	130/166	8	0.67	10	0.66	10	0.66	8	0.47	4	0.62
CH01H10	91/119	8	0.73	8	0.70	9	0.71	12	0.83	6	0.79
CH02D08	206/254	11	0.88	13	0.87	14	0.87	10	0.83	8	0.88
CH02C02b	75/123	4	0.59	7	0.64	8	0.62	3	0.44	2	0.47
CH02C06	218/265	15	0.86	16	0.89	17	0.88	13	0.89	6	0.78
Mean		10.5	0.79	12.4	0.80	13.5	0.80	9.7	0.76	6.3	0.77

Table 5. F-statistics (Weir and Cockerham, 1984; estimated with SPAGeDI 1.2) for 71 apple accessions from Sarajevo and eastern Bosnia and for all 108 analyzed accessions (reference cultivars included) based on 10 simple sequence repeat loci.

	Apple accessions from Sarajevo and eastern Bosnia (N = 71)						Apple accessions from Sarajevo and eastern Bosnia together with reference cultivars from ex situ collection Srebrenik (N = 108)					
Locus code	Fit	*P*	Fis	*P*	Fst	*P*	Fit	*P*	Fis	*P*	Fst	*P*
CH01H01	–0.106	<0.0001	–0.100	<0.0001	–0.005	0.314	–0.080	0.005	–0.077	0.007	–0.003	0.662
CH05E03	0.182	<0.0001	0.186	<0.0001	–0.005	0.628	0.198	<0.0001	0.190	<0.0001	0.010	0.077
CH05E04	–0.035	0.497	–0.050	0.346	0.015	0.061	0.014	0.730	–0.010	0.829	0.023	<0.0001
CH01H02	–0.145	0.003	–0.135	0.013	–0.009	0.135	–0.078	0.095	–0.108	0.030	0.027	0.009
CH02C02a	–0.019	0.535	–0.030	0.272	0.011	0.051	–0.010	0.656	–0.031	0.216	0.021	<0.0001
CH04E02	–0.009	0.934	–0.002	0.999	–0.007	0.482	0.012	0.806	0.002	0.890	0.010	0.209
CH01H10	–0.159	0.007	–0.151	0.008	–0.008	0.241	–0.119	0.003	–0.131	<0.0001	0.011	0.095
CH02D08	–0.080	0.037	–0.078	0.026	–0.002	0.886	–0.066	0.035	–0.072	0.015	0.006	0.227
CH02C02b	–0.081	0.343	–0.113	0.159	0.029	0.061	0.075	0.305	0.013	0.819	0.063	0.003
CH02C06	–0.066	0.025	–0.073	0.032	0.006	0.203	–0.038	0.153	–0.068	0.009	0.029	<0.0001
ALL LOCI	–0.047	<0.0001	–0.050	<0.0001	0.002	0.391	–0.009	0.459	–0.028	0.025	0.019	<0.0001

previous studies, which examined differentiation between traditional and international apple cultivars in Bosnia and in Spain (Gasi et al., 2010; Pereira-Lorenzo et al., 2006, 2008). Further examination of genetic differences among the four groups of apple accessions was performed using AMOVA. AMOVA calculated for all 108 analyzed accessions indicated that most of the variance was retained within the groups of accession (99%), whereas 1% of the total diversity was attributed to the differences among the analyzed groups of accessions (Table 6). The largest percentage of variance between the groups was detected among apple accessions from Sarajevo and international reference cultivars ($f_{CT} = 0.051$; $P < 0.01$) as well as among apple accessions from eastern Bosnia and international reference cultivars ($f_{CT} = 0.058$; $P < 0.01$).

The smallest percentage of variance between the groups was detected among apple accessions from Sarajevo and eastern Bosnia ($f_{CT} = 0.001$; *P* value nonsignificant), which is in concordance with the low Fst values calculated for these groups. Nonsignificant f_{CT} was also calculated among apple accessions from eastern Bosnia and traditional B&H reference cultivars, indicating that both of the groups, at least in part, belong to the same germplasm. Although the percentage of variance among apple accessions from Sarajevo and traditional B&H reference cultivars

Table 6. Analysis of molecular variance (AMOVA) based on the 10 simple sequence repeat loci of 71 apple accessions corresponding to two areas in Bosnia and Herzegovina (B&H) (Sarajevo and eastern Bosnia) as well as 24 traditional B&H and 13 international reference apple cultivars from the ex situ collection Srebrenik and to the two reconstructed populations (RPPs) defined by Structure (Pritchard et al., 2000).

Source of variation	df	Variance components	Total variance (%)	f_{CT}	*P*
All 108 analyzed accessions (reference cultivars included)					
Among groups	104	78.95	99.0	0.010	<0.01
Within groups	3	0.79	1.0		
Total	107	79.74			
Apple accessions from Sarajevo and eastern Bosnia					
Among groups	69	80.61	99.9	0.001	0.3720
Within groups	1	0.10	0.1		
Total	70	80.71			
Apple accessions from Sarajevo and international reference cultivars					
Among groups	42	75.80	94.9	0.051	<0.01
Within groups	1	4.10	5.1		
Total	43	79.91			
Apple accessions from Sarajevo and traditional B&H reference cultivars					
Among groups	53	78.04	97.1	0.029	<0.05
Within groups	1	2.31	2.9		
Total	54	80.35			
Apple accessions from eastern Bosnia and international reference cultivars					
Among groups	51	79.96	94.2	0.058	<0.01
Within groups	1	4.89	5.8		
Total	52	84.85			
Apple accessions from eastern Bosnia and traditional B&H reference cultivars					
Among groups	62	81.24	98.8	0.012	0.0940
Within groups	1	0.96	1.2		
Total	63	82.20			
RPPs					
Among RPPs	104	74.98	87.0	0.130	<0.001
Within RPPs	1	11.17	13.0		
Total	105	86.15			

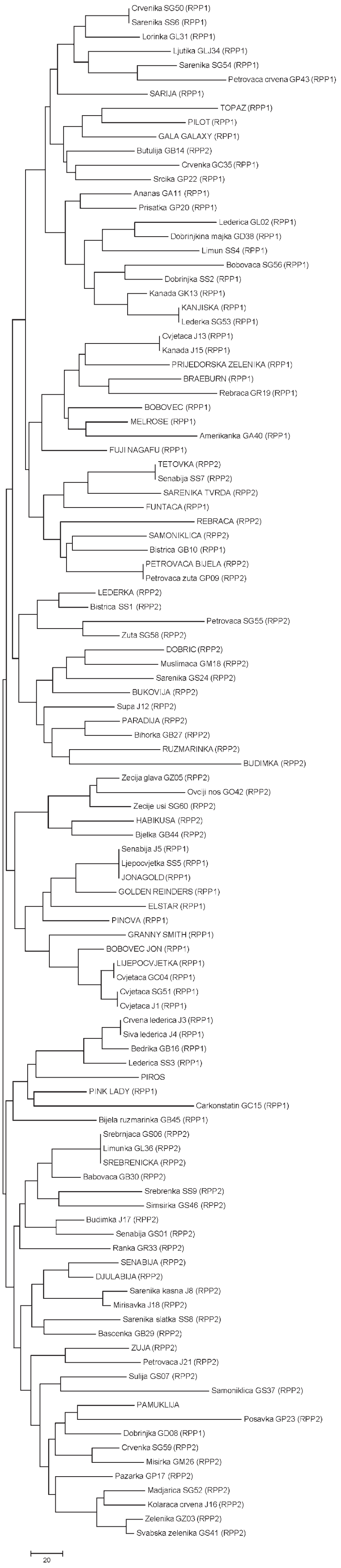

Fig. 1. Neighbor joining cluster analysis based on polymorphisms of 10 simple sequence repeat loci in 71 local apple cultivars and 37 reference apple cultivars (written in capital letters) using Bruvo genetic distance (Bruvo et al., 2004). In brackets are the reconstructed populations (RPP1 or RPP2) to which each cultivar is assigned with probability of membership *qI* greater than 80%.

was low (2.9%), it was significant ($P < 0.05$), indicating that a significant part of Sarajevo accessions belongs to the international apple germplasm.

Genetic relationships. Bayesian analyses, which were done within Structure, revealed that almost all of the international reference cultivars (92%) grouped in RPP1, whereas most traditional B&H reference cultivars (71%) were in RPP2. Local apple cultivars from Sarajevo and eastern Bosnia grouped in both RPPs. The first RPP included 53 genotypes, all of them with *qI* greater than 80%. Of those, 12 were international reference cultivars, seven traditional B&H reference cultivars, 16 apple accessions from Sarajevo, and 18 accessions from eastern Bosnia. The second RPP included 55 genotypes, 53 of them with *qI* greater than 80%. Of those, 16 were traditional B&H reference cultivars, 15 apple accessions from Sarajevo, and 22 accessions from eastern Bosnia. Overall, approximately half of the local apple cultivars from Sarajevo and eastern Bosnia (52% and 45%, respectively) grouped in the RPP consisting mainly out of international cultivars, whereas the other half grouped in the RPP with traditional B&H reference cultivars. Structure analysis therefore confirmed the conclusion based on the AMOVA results, that the local accessions analyzed in this study represent a mixture of traditional B&H and international germplasm. The variance among RPPs (13%; $P < 0.001$) (Table 6) was significant, although slightly lower than reported by Pereira-Lorenzo et al. (2008) (14.41%) for RPPs constructed with Spanish and international cultivars but higher than the values obtained by Gasi et al. (2010) (9.2%) in a previous study on apple germplasm in B&H.

NJ cluster analysis was used to group all 108 examined accessions (Fig. 1). The tree was constructed based on a genetic distance model, which takes into consideration mutation characteristics of microsatellites and allows genotypes with different ploidy level to be included in analyses (Bruvo et al., 2004). Although the separation of most of the traditional B&H and international reference cultivars was evident, accessions from Sarajevo and eastern Bosnia were dispersed throughout the entire dendrogram. A large number of the mentioned accessions formed clusters exclusively with traditional B&H reference cultivars, whereas a slightly smaller number grouped very tightly with international reference cultivars indicating introgression of foreign genotypes into the local germplasm and/or presence of mislabeled international cultivars. In Figure 1, all cultivars with *qI* over the minimum threshold of 80% were assigned to one of the two RPPs. It is interesting to note that although NJ cluster analysis based on Bruvo genetic distance represents a different biostatistical approach than the Bayesian model-based cluster procedure in Structure, the results of both analyses largely concurred. Namely, only three accessions from the RPP1 clustered tightly with genotypes from RPP2, whereas only one accession from RRP2 grouped in a cluster containing RPP1 accessions (Fig. 1).

To get a clearer picture of the relationships between the analyzed groups of accessions, a multivariate approach based on FCA analysis was used. The three-dimensional plot (Fig. 2) revealed a clear differentiation between the traditional B&H and international reference cultivars, whereas the groups of accessions from Sarajevo and eastern Bosnia overlapped. Because the genetic differentiation between these groups is negligible (nonsignificant values for Fst and *P* in AMOVA), this was completely expected. Apple accessions from both Sarajevo and eastern Bosnia generally clustered closer to traditional than to the international reference cultivars, whereas some overlapping between eastern Bosnia and traditional B&H reference group was noted. Both of these observations are in concordance with the results of ANOVA (Table 6). Factorial correspondence analysis was also done for the two RPPs and it revealed a strong genetic differentiation between RPP1 and RPP2 (Fig. 3), which is in concordance with the results of AMOVA.

Conclusions

Molecular data indicated that both groups of apple accessions analyzed (from Sarajevo and from eastern Bosnia) represent an interesting source of diversity, which needs to be conserved. Although introgression of international reference cultivars in local apple germplasm was detected, both groups contain a large number of diverse traditional B&H apple cultivars, which have so far not been preserved, and inclusion of all this material in the ex situ collection is imperative. In this process, priority should be given to all the local apple accessions from Sarajevo and eastern Bosnia, which grouped in the RPP2. Accessions maintained in the ex situ collection can then be investigated for advantageous agronomic traits.

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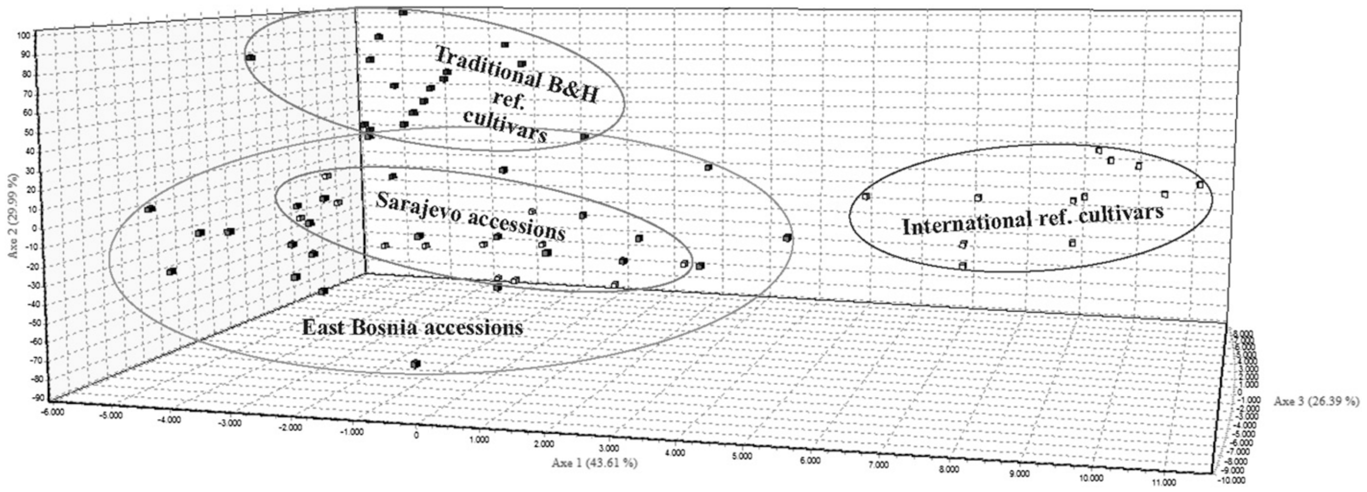

Fig. 2. Multivariate analysis (factorial correspondence analysis) of simple sequence repeat data for 37 apple accessions from two areas in Bosnia and Herzegovina (B&H) (13 from Sarajevo and 24 from eastern Bosnia) as well as 19 traditional B&H and 12 international reference apple cultivars from ex situ collection Srebrenik (diploids analyzed only).

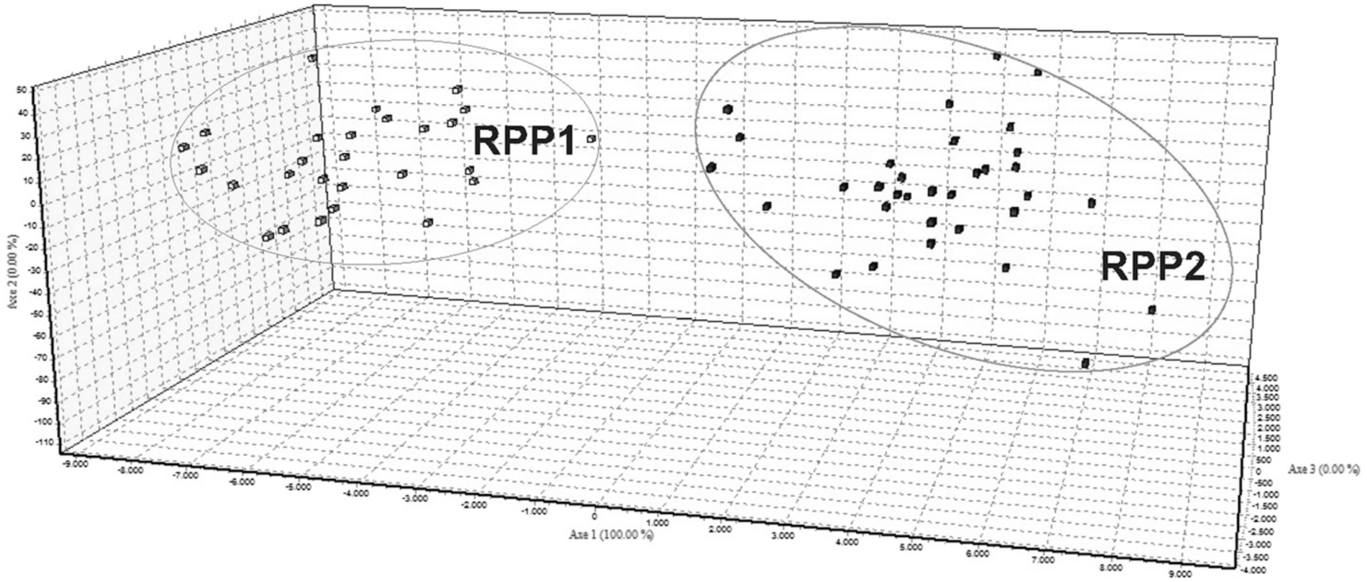

Fig. 3. Multivariate analysis (factorial correspondence analysis) of simple sequence repeat data for reconstructed populations (RPPs) calculated using Structure (Pritchard et al., 2000) (only diploid genotypes with *qI* greater than 80%). Ninety-two percent of the international reference cultivars grouped in RPP1, whereas 71% of traditional Bosnia and Herzegovina (B&H) reference cultivars were in RPP2. Local apple cultivars from Sarajevo and eastern Bosnia grouped in both RPPs.

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