Journal of Applied Botany and Food Quality 85, 229 - 233 (2012)

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Simple sequence repeat (SSR) analysis for assessment of genetic variability in wild cherry germplasm

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(Received October 22, 2012)

Summary

Conservation of genetic resources is vital for future breeding programs and food security for humans. Before conservation of genetic resources, it requires objective characterization and a proper assignation of individual genotypes to species. The aim of this study was to characterize of 58 *Prunus* accessions which belong to *Prunus avium*, *Prunus cerasus* and *Prunus mahaleb* by using 12 SSR markers. All twelve SSR markers produced successful amplifications and revealed DNA polymorphisms. The number of allele per loci varied from 6 (UDP96-019) to 12 (PS12A02) with an average of 9 per allele. The average of observed and expected heterozygosity was found to be 0.609 and 0.720. The allele size varied from 95 to 276 bp. The number of genotypes per allele were 7 (UCD-CH13) and 24 (UDP96-005). Genetic distance analysis based on SSRs divided the cherry accessions in three main groups based mainly on their species characteristics. *P. cerasus* genotypes had higher similarity ratio within species than *P. mahaleb* and *P. avium*.

Introduction

Sweet cherry, sour cherry and mahaleb belongs to *Rosaceae* family, *Prunoideae* subfamily, *Prunus* genus and *Cerasus* subgenus. *Cerasus* is further divided into four groups including *Eucerasus*, *Microcerasus, Pseudocerasus* and *Mahaleb*. Sweet cherries (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.) are placed in *Eucerasus* group while mahleb (*Prunus mahaleb* L.) is placed in *Mahaleb* group (Ercisli, 2004).

Asia minor in Turkey is one of the origins and domestication centers for *P. avium, P. cerasus and P. mahaleb* (Ozcagiran et al., 2005). *P. avium* is originated in the area between the Black and Caspian seas of Asia minor. Great morphological variation exists among *P. avium*, *P.mahaleb* and *P. cerasus* accessions naturally grown as wild in Turkey (Ercisli, 2004). This continuum of morphological characteristics makes species assignation difficult when considering only phenotypic traits. In Turkey *Prunus mahaleb*, wild *Prunus avium* and *Prunus cerasus* seedlings commonly have been used as rootstocks for both sweet and sour cherry cultivars (Ercisli et al., 2011).

Conservation of genetic resources is vital for future breeding programs and food security for humans. Before conservation of genetic resources, objective characterization and a proper assignation of individual genotypes to species is required (Karp et al., 1997).

Among the different marker systems (Morphological, Biochemical and Molecular), molecular markers supply more reliable tools to analyze genetic diversity in plant species (Karp et al., 1997). They could be helpful by giving an accurate and unambiguous assignation of each genotype to a particular species (Szikriszt et al., 2011).

Simple sequence repeats (SSRs or microsatellites) have become the genetic markers of choice in many plant species because they are PCR-based, highly reproducible, polymorphic, generally codominant and abundant in plant genomes (Powell et al., 1996). SSR loci can be identified and their alleles recognized in different genotypes of the same species because of their codominant and usually single-locus nature, and often in those of other close relatives. In other words a specific set of SSRs can be used in different sets of genotypes, making them particularly useful for fingerprinting. In general, SSRs are more transferable between species of the same genus, or between closely related genera, than between distant genera of the same family (Peakall et al., 1998; Zhang et al., 2005; Hendre et al., 2008; Luro et al., 2008).

Previously SSR (Simple Sequence Repeats) markers have been successfully used on genotypes belong to different *Prunus* genus in diversity studies (Cheng and Huang, 2009; Guarino et al., 2009; Lacis et al., 2009; Gulen et al., 2010; Nas et al., 2011). However, the majority of these studies have dealt with cultivars and little emphasis has been paid to wild relatives. This study has therefore sought to document indigenous knowledge related to uses of wild grown genotypes which belong to *P. avium, P. cerasus* and *P. mahaleb*.

Materials and methods

Plant material

For SSR and genetic relationship studies, 37 *Prunus avium*, 8 *Prunus cerasus* and 7 *Prunus mahaleb* rootstock candidates together with well-known standard rootstocks of each species, F12/1 (*Prunus avium* L.), Montmorency (*Prunus cerasus*) and SL 64 (*Prunus mahaleb* L.) were used. These genotypes have been previously selected from wild cherry populations as rootstock candidates in Ordu region in Turkey. All genotypes are maintained in a germplasm collection at the Black Sea Agricultural Research Center in Samsun, Turkey.

DNA extraction

Genomic DNA was extracted from young leaf tissue using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the instructions provided by the manufacturer. Subsequently, an RNAse treatment was performed on the eluted DNA samples. Purity and concentration of the DNA were checked both on 1% (w/v) agarose gels and by NanoDrop® ND-1000 Spectrophotometer.

SSR analysis

From an initial screening, 12 SSRs were selected to check for polymorphism by capillary electrophoresis in 55 genotypes of three different *Prunus* species (Tab. 1). **Polymerase Chain Reaction** (PCR) was conducted in a volume of 10 µL and contained 15 ng genomic DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5 unit GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 2 µL 5X buffer. The forward primers were “labelled” with WellRED fluorescent dyes D2 (black), D3 (green) and D4 (blue) (Proligo, Paris, France). Reactions without DNA were included as negative

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controls. PCR amplification was performed using the Biometra® PCR System. The amplification conditions consisted of an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52-56°C and 2 mins at 72°C with a final extension at 72°C for 10 mins. The PCR products were first separated on a 3% (w/v) agarose gel run at 80 V for 2 hrs. The gel was then stained with ethidium bromide at a concentration of 10 mg/mL. A DNA ladder (100 bp) (Promega) was used for the approximate quantification of the bands. The amplification products were visualized under UV light, and their sizes were estimated relative to the DNA ladder. For further determination of polymorphisms, the PCR products were run on CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA). The analyses were repeated at least twice to ensure reproducibility of the results. Allele sizes were determined for each SSR locus using the Beckman CEQTM fragment analysis software. In each run, SL64, F12/1 and Montmorency were included as reference rootstocks.

Genetic analysis

The genetic analysis program "IDENTITY" 1.0 (Wagner and Sefc, 1999) was used according to Paetkau et al. (1995) for the calculation of number of alleles, allele frequency, expected and observed heterozygosity, estimated frequency of null alleles, and probability of identity per locus. Genetic dissimilarity was determined by the program "MICROSAT" (version 1.5) (Minch et al., 1995) using proportion of shared alleles, which was calculated by using "ps (option 1 - (ps))", as described by Bowcock et al. (1994). The results were then converted to a similarity matrix, and a dendrogram was constructed with the UPGMA method (Sneath and Sokal, 1973) using the software NTSYS-pc (Numerical Taxonomy and Multiware Analysis System,version 2.0) (Rohlf, 1988).

Results and discussion

The total 12 SSRs studied amplified 108 alleles, an average of 9 alleles per locus in 58 *Prunus* accessions belongs to *Prunus avium*, *Prunus cerasus* and *Prunus mahaleb*. The highest number of allele per primer was observed in PS12A02 primer as 12 alleles and followed by Pchgms1, UDP96-001 and UDP96-005 primers (11 alleles). The primer UDP96-019 gave the lowest number of alleles (6 alleles) (Tab. 1).

We observed an average SSR observed heterozygosity (Ho) of 0.609 and the observed heterozygosity were found between 0.345 (Pchgms1) and 0.890 (UCD-CH31). The average expected heterozygosity (He) was 0.720 indicating higher value than observed one (Tab. 1).

The probability of genetic identity (PI) was the lowest in PS12A02 locus (PI:0.089) indicating that this loci was the most informative while the locus UDP96-019 (PI: 0.373) was the less informative.

The allele sizes of 12 SSR locus varied from 95 to 276 bp. The number of genotypes per allele were between 7 (UCD-CH13) and 24 (UDP96-005) (Tab. 2).

The genetic similarity measured within species ranged between 0.17-0.79 within *P. avium*, 0.96-1.00 within *P. cerasus*, 0.58-0.83 within *P. mahaleb* genotypes. The average similarity ratios within species in a descending order were *P. cerasus* (0.98)> *P. mahaleb* (0.72)> *P. avium* (0.51), respectively. The similarity ratio were 0.25-0.58 between *P. avium* accessions and F12/1; *P. cerasus* accessions and Montmorency was 0.63 and *P. mahaleb* accessions and SL64 were 0.46-0.63, respectively. The average similarity ratio between *P. avium-P. cerasus; P. avium-P. mahaleb* and *P. cerasus-P. mahaleb* were 0.33; 0.007 and 0.08, respectively, indicating *P. avium* is more close to *P. cerasus*.

A tree constructed from the SSR data divided the accessions into 3 main clusters according to their taxonomic classification. The first cluster included *P. avium* accessions, the second cluster included *P. cerasus* accessions and the last cluster included *P. mahaleb* accessions (Fig. 1). *P. cerasus* accessions seem to be identical or very closely related. In contrast to *P. cerasus*, *P. mahaleb* and in particular *P. avium* seem to be more differentiated. The reference rootstocks were also clustered with their associated botanical species (Fig. 1).

Tab. 1: List of genetic parameters obtained with SSR used in this study

Locus	N	He	Ho	PI	R
CPSCT010	10	0.648	0.581	0.190	0.040
Pchgms1	11	0.723	0.345	0.142	0.219
PS12A02	12	0.806	0.636	0.089	0.094
UCD-CH13	7	0.697	0.836	0.237	-0.081
UCD-CH17	8	0.861	0.418	0.068	0.238
UCD-CH21	8	0.697	0.381	0.175	0.186
UCD-CH31	8	0.773	0.890	0.146	-0.006
UDAp-401	8	0.729	0.636	0.177	0.053
UDAp-404	8	0.606	0.818	0.373	-0.131
UDP96-001	11	0.807	0.545	0.084	0.144
UDP96-005	11	0.850	0.818	0.072	0.017
UDP96-019	6	0.440	0.400	0.370	0.028
Total	133				
Average	13.3	0.81	0.57		

N: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PI:probability of genetic identity; r: null allele frequencies

The results obtained in the present study show that microsatellites could be effectively used for fingerprinting purposes in *Prunus*. In the present study, 12 loci in wild *Prunus* genotypes were assayed. The number of alleles per locus ranged from 6 to 12 with an average of 9 putative alleles per locus. Previously, Kacar et al. (2005) obtained a total of 37 alleles among 10 sweet cherry cultivars by 9 SSR primers. Clarke and Tobutt (2003) used 14 sweet cherry cultivars for SSR analysis and determined 2 to 7 alleles per SSR primer. In addition, Vaughan and Russell (2004) used 16 wild cherry accessions for molecular analysis by using 10 SSR primers and they detected 2 to 6 alleles. In fact all tested microsatellite primer pairs worked well and produced variable levels of amplifications. The PS12A02 locus was the most polymorphic among the twelve loci with the highest effective number of alleles (12 alleles) with the one of the lowest PI value (0.089). The UDP96-019 was the less informative with the lowest allele number (6). The results showed high amplification of cherry groups with plum, apricot and peach indicating a congeneric relationship within *Prunus* species. Ercisli et al. (2011) successfully used SSR markers identified in other *Prunus* species to study genetic diversity in wild sweet cherries. Our results demonstrated the cross-species transferability of SSR primers developed in cultivated species to wild species in *Prunus* for the discrimination of genotypes. Previously, PS12A02 loci were found to be the most informative in other studies (Downey and Iezzoni, 2000; Gulen et al., 2010; Ercisli et al., 2011). Wünsch et al. (2004) reported 7 and 11 alleles in UDP96-005 and Pchgms1 locus in sweet cherries. In our study, UDP96-019 and UCD-CH13 gave the lowest number of alleles (6 and 7). Struss et al. (2003) and Zhang et al. (2008) obtained the lowest allele in UCD-CH31 loci and Wünsch and Hormaza (2002) reported the lowest loci in UDP96-019 loci.

The overall genetic diversity within the tested species was relatively low as evident from the polymorphic ratio of 21% found by SSR

Tab. 2: The allele sizes (bp) of *Prunus* accessions at 12 SSR locus

Genotype No	PS12A02	UCD-CH17	Pchgms1	UDAp-401	UCD-CH31	UCD-CH21	UDAp-404	UDP96-001	UDP96-005	CPSCT010	UCD-CH13	UDP96-019
P. avium												
37	158:166	202:202	138:138	262:262	130:142	111:119	154:168	115:123	109:115	176:176	122:136	202:202
38	158:174	202:202	130:130	262:262	124:142	111:111	154:168	123:123	115:119	176:176	126:136	202:202
39	158:158	200:200	130:136	262:262	130:142	109:109	154:168	105:125	125:125	180:180	126:136	202:202
40	158:158	198:198	130:138	260:266	130:142	107:107	154:168	109:123	109.125	176:176	126:136	202:202
41	158:166	198:198	142:152	262:262	130:142	109:109	154:162	123:133	115:119	176:176	126:136	202:202
42	158:166	180:198	138:138	262:266	130:142	107:111	154:168	123:123	125:135	176:176	126:136	202:202
43	158:174	198:198	152: 152	262:266	132:142	109:109	154:168	123:123	109:109	176:180	136:136	202:202
44	154:174	180:202	138:138	262:266	130:142	109:109	154:168	105:105	119:119	176:176	126:136	202:202
45	158:166	190:204	136:136	262:262	130:142	109:109	154:162	123:123	109:119	176:180	122:136	202:202
46	158:166	202:202	138:138	262:262	130:142	109:109	154:162	109:115	109:115	176:176	126:136	202:202
47	158:158	180:200	138:158	262:266	124:130	109:109	154:168	109:123	109:119	176:176	126:136	202:202
48	160:174	190:190	140:140	266:266	124:142	109:109	168:168	123:123	119:135	176:176	132:136	202:202
49	154:160	190:190	140:140	260:260	128:142	109:109	168:168	105:125	115:119	174:178	126:136	202:204
50	174:174	200:200	140:140	262:266	132:142	109:109	154:162	123:123	115:119	176:176	126:136	202:202
51	158:158	190:190	130:138	262:266	130:142	111:111	154:168	123:123	109:119	176:176	126:136	202:202
52	158:158	180:200	138:138	262:266	124:130	109:117	154:168	105:123	119:133	176.182	126:136	202:202
53	158:166	190:204	130:158	262:266	132:142	107:119	154:162	115:123	133:143	176:180	122:136	202:202
54	174:174	188:198	130:152	262:262	130:142	109:109	154:162	123:123	119:133	176:176	126:136	202:202
55	154:158	190:190	138:138	262:262	130:142	109:109	154:162	123:137	109:119	176:180	126:136	202:202
56	166:166	200:200	138:138	270:270	130:142	107:111	154:162	105:125	109:115	176:180	126:136	202:204
57	160:174	198:198	138:138	262:272	130:142	109:109	154:162	123:123	115:135	176:176	126:136	202:202
58	158:166	198:198	138:138	262:262	130:142	111:115	154:162	123:123	115:115	176:176	126:136	204:204
59	158:174	202:202	130:140	270:270	130:142	111:111	154:162	105:125	115:115	176:176	126:136	202:204
60	158:158	180:180	138:138	262:262	124:130	109:109	154:162	105:123	115:115	180:180	126:136	182:202
61	154:158	198:198	138:138	262:266	130:142	109:109	162:168	109:115	115:125	176:176	122:136	202:202
62	162:162	200:200	138:138	262:262	132:142	111:119	154:162	123:123	143:143	176:176	136:144	202:204
63	158:166	198:198	138:138	262:262	130:142	111:119	154:168	109:125	119:125	174:176	126:136	182:204
64	158:166	180:202	138:138	262:272	130:142	109:109	154:162	125:137	115:133	176.176	126:136	182:202
65	158:158	200:200	130:136	260:272	128:142	109:109	164:168	123:123	115:133	176:176	126:136	202:202
66	160:174	180:200	146:146	260:266	128:142	109:109	154:162	109:123	109:115	176:180	126:136	202:202
67	158:158	188:204	138:158	266:272	130:142	109:119	154:162	123:123	135:135	176:180	126:136	202:202
68	154:158	188:200	138:138	262:266	132:142	107:111	154:162	105:125	109:125	176:180	126:136	202:202
69	158:158	202:202	138:138	262:262	130:142	109:109	154:162	109:115	115:135	176:180	122:136	202:202
70	158:158	188:204	138:138	262:262	124:130	109:119	168:178	123:123	109:115	176:176	126:136	202:202
71	158:174	190:198	138:138	266:266	130:142	109:109	168:168	115:125	115:115	174:182	126:136	182:202
72	158:158	200:200	138:138	266:272	130:142	109:109	154:162	123:123	109:115	174:176	126:136	202.202
73	158:158	188:200	130:130	260:266	130:142	109:119	154:162	123:133	115:119	176:180	126:136	202:202

Genotype No	PS12A02	UCD-CH17	Pchgms1	UDAp-401	UCD-CH31	UCD-CH21	UDAp-404	UDP96-001	UDP96-005	CPSCT010	UCD-CH13	UDP96-019
P. cerasus												
122	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
123	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
124	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
125	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
126	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
127	146:160	180:188	138:160	266:276	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
128	146:160	180:188	138:160	266:276	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
186	146:160	180:188	138:160	262:266	124:132	103:109	154:168	99:113	109:119	170:176	128:136	190:202
P. mahaleb												
152	162:162	164:164	188:188	138:146	100:100	95:95	168:168	115:115	113:119	212:232	122:122	202:208
153	156:166	164:164	188:188	138:146	106:106	95:95	168:168	117:117	113:123	212:232	122:122	202:208
154	162:162	164:164	188:188	138:146	106:106	95:95	170:170	117:117	113:119	212:232	122:122	202.208
155	146:164	164:164	188:188	138:146	124:124	95:95	170:170	117:117	113:123	212:232	122:122	202:208
156	164:172	164:164	188:188	138:146	100:100	95:95	168:172	117:117	113:123	212:232	122:122	202:218
157	162:162	164:164	188:188	138:146	100:122	95:95	170:170	115:115	113:117	212:232	122:122	202:202
158	146:162	164:164	188:188	138:146	100:122	95:95	168:168	115:115	113:123	212:232	122:122	208:208
SL64 (189)	142:142	164:164	188:188	138:146	124:124	95:95	168:168	119:119	113:113	206:228	122:122	208:208
F12/1 (201)	156:176	202:202	138: 138	262:272	124:130	103:103	154:162	123:123	127:135	182:182	126:136	202:202
MM (202)	158:164	180:192	138:162	266:266	122:130	103:109	154:168	99:113	103:115	170:176	126:136	190.202
Allele size	142-174	164-204	130-188	138-276	100-142	95-119	154-178	99-137	103-143	174-232	122-136	190-218
Allele per genotype	17	16	15	11	11	12	8	16	24	11	7	9

MM:Montmorency

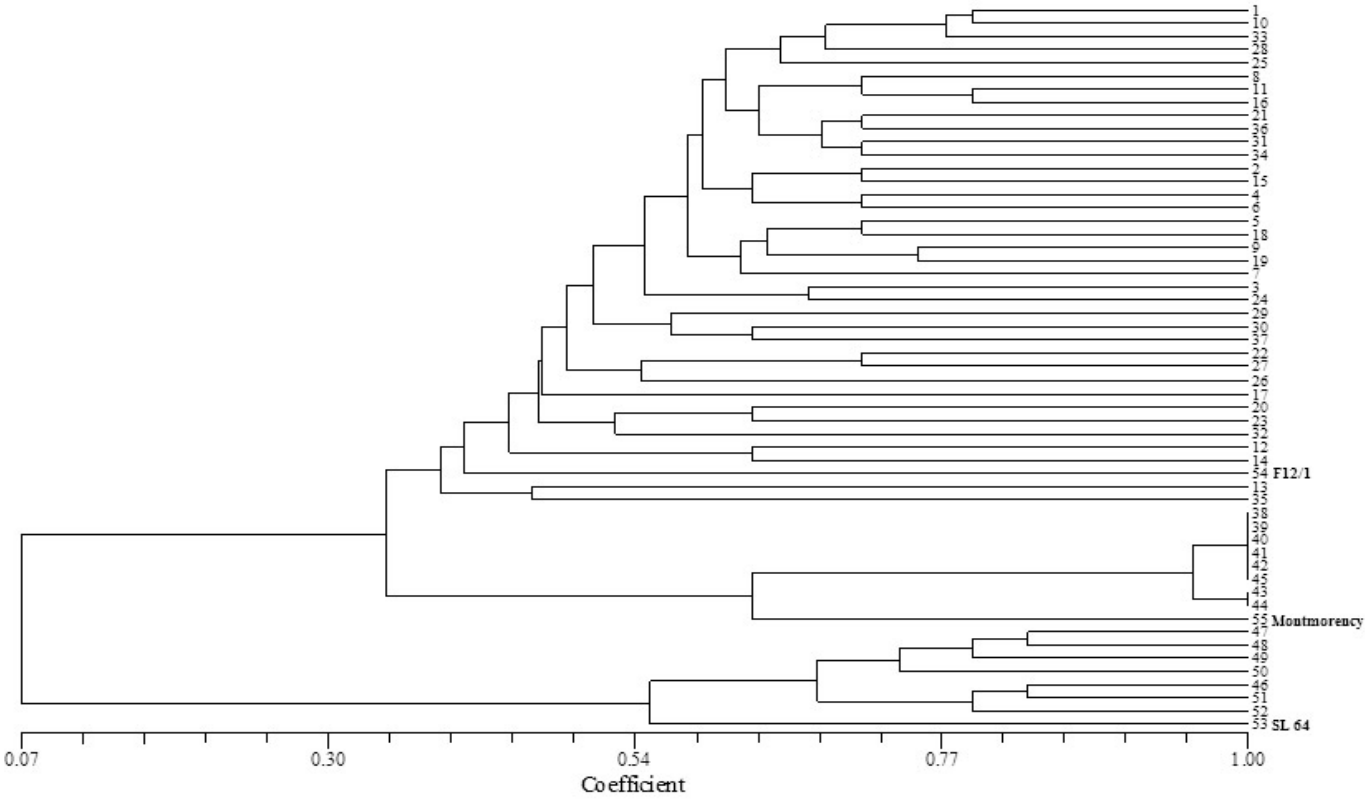

Fig. 1: Dendrogram of 58 *Prunus* accessions based on UPGMA analysis using the genetic similarity matrix generated by the Nei and Li similarity coefficient after amplification with 12 pairs of microsatellite primers.

primers (STRUSS et al., 2003) and 19% reported by ZHOU et al. (2002) and in cherries. We found high diversity ratio within *P. avium* compared to other species. A higher level of polymorphism was expected in sweet cherry due to its predominant self-incompatibility (HEGEDUS et al., 2012).

The observed and expected heterozygosities averaged over the 12 SSR loci were respectively 0.61 and 0.72 indicating higher mean values than those reported for SSRs in *Prunus* species (ARANZANA et al*.,* 2003; BOUHADIDA et al*.,* 2009). High allele number and high heterozygosity obtained in the present study reflect the ability of SSR markers to provide unique genetic profile for individual plant accessions, except in *P.cerasus* accessions. VAUGHAN and RUSSELL (2004) reported He and Ho values as 0.61 and 0.60 in 16 wild sweet cherry accessions by using 14 SSR locus.

Conclusion

In conclusion, the gene pool of the *Prunus* species surveyed in Black Sea and Northeast Anatolia has significant amounts of genetic variation. In regard to germplasm management, our results show that the germplasm collection is highly variable and most variation is common to all genetic groups identified. *Prunus* germplasm from the region would have economically important adaptive traits that can potentially be incorporated into *Prunus* breeding programs. Hence, it is expected that the results of this study will assist current *Prunus* rootstock breeding efforts in Turkey as well as maintain the genetic integrity of the genetic resources. The SSR-based phylogeny was also generally consistent with *Prunus* taxonomy based on molecular evidence, suggesting the applicability of SSR analysis for genotyping and phylogenetic studies in *Prunus* genus.

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