

Chloroplast Microsatellite Diversity Among and Within *Prunus mahaleb* L. and *P. avium* L. Species

Masoud Abedian

Department of Agricultural Biotechnology, College of Agriculture
Isfahan University of Technology, Isfahan 84156 83111, Iran

Majid Talebi (Corresponding author)

Department of Agricultural Biotechnology, College of Agriculture
Isfahan University of Technology, Isfahan 84156 83111, Iran
Tel: 98-311-391-3345 E-mail: mtalebi@cc.iut.ac.ir

Badraddin-Ebrahim Sayed-Tabatabaei & Cyrus Ghobadi

Department of Agricultural Biotechnology, College of Agriculture
Isfahan University of Technology, Isfahan 84156 83111, Iran

Received: December 26, 2011

Accepted: January 9, 2012

Online Published: April 1, 2012

doi:10.5539/jas.v4n5p191

URL: <http://dx.doi.org/10.5539/jas.v4n5p191>

Abstract

Genetic diversity of 58 Mahaleb cherry (*Prunus mahaleb*) genotypes and six sweet cherry (*P. avium*) accessions was studied using 25 cpSSR primer pairs. Thirteen out of them demonstrated two to four alleles with an average of 2.46 alleles per primer pair. The mean of PIC value for the primers was 0.32. The average values of expected heterozygosity (*He*) and Shannon's information index (*I*) for all loci were 0.35 and 0.55, respectively. The dendrogram based on cpSSR markers has been illustrated by MEGA4 software with Maximum Composite likelihood model and the Neighbor-joining method, which clustered the genotypes into four groups. Sum of first three principal components analysis (PCAs) could be represented most of (63.07%) the total variation in the original dimensions and confirmed the results of cluster analysis. Based on the AMOVA results, the allele numbers among groups and species studied in this research were more than those observed within them. In this study cpSSR markers provided a good tool for assessment of genetic diversity among and within mahaleb cherry and sweet cherry genotypes.

Keywords: Mahaleb cherry, Sweet cherry, Genetic diversity, cpSSR, AMOVA

1. Introduction

Prunus is a large genus of trees and shrubs, which includes plums, cherries, peaches, apricots, and almonds. Botanical classification of species within this genus, partly due to ease of interspecific hybridization is sometimes debatable (Turkoglu *et al.*, 2010). These species belong to the Rosaceae, sub family Prunoidae. There are many different types of rootstocks being used for *Prunus* species. Each one has a particular set of advantages and limitations for adaptation to different geographic regions. One of the *Prunus* species used as a common rootstock for sweet cherry that has been known as a cultivar with strong roots, is Mahaleb cherry (*P. mahaleb* L.), ($2n = 2x = 14$). This species is native to Mediterranean, Southeast Europe and West Asia, however, it is sometimes found in Central Europe. Another *Prunus* species is Sweet cherry (*P. avium* L.), ($2n = 2x = 16$). This species is typical outcrossing with a mono-factorial and multi-allelic gametophytic incompatibility system (Crane and Lawrence 1929; Lacis *et al.*, 2009; Tehrani and Brown 1992). Sweet cherry is one of spring-summer fruit species which is consumed as a fresh fruit (Jakobek *et al.*, 2009). The available genetic diversity of species such as Mahaleb cherry and Sweet cherry can improve breeding of these species.

The genomic studies concerning the fruit species have increased enormously to characterize fruit germplasm resources and analysis of their genetic diversity including *P. mahaleb* and *P. avium* species based on morphological characteristics and molecular markers (Ganji Moghadam and Khalighi 2006; Lacis *et al.*, 2009; Pedersen 2006; Rakonjac *et al.*, 1996; Wunsch and Hormaza 2002 and 2004). For crop improvement studies, researchers usually request plentiful genetic diversity among materials.

For breeding and commercialization of rootstocks, a precise determination and discrimination method for these materials is desired. Morphological traits are strongly affected by the environment and developmental stage of plants (Casas *et al.*, 1999). Therefore, it is very difficult to follow their morphological traits of rootstocks after grafting. Molecular markers are useful complements to morphological and phenotypic characters because they are plentiful, independent of tissue or environmental effects, and allow reliable identification and discrimination of genotypes in the early stages of development. The superiority of molecular markers over morphological characterization in fruit species is well recognized and widely accepted (Duminil and Di Michele, 2009; Ercisli *et al.*, 2007; Zamani *et al.*, 2007).

The chloroplast simple sequence repeat (cpSSR) is a DNA-based molecular marker with multi-allelic nature, co-dominant and reliable PCR-based marker system. The existence of highly polymorphic simple sequence repeats (SSRs) in the chloroplast genome of plants has provided opportunities to determine genetic diversity in population studies (Powell *et al.*, 1995, 1996). Several researches have been reported on the genetic variation within and among species using cpSSR markers (Malay *et al.*, 2009; Mehes *et al.*, 2009; Yan *et al.*, 2009). The cpSSR markers have been widely applied for researches on plant population genetics, phylogenetics, as well as germplasm identification and resource conservation (Dai *et al.*, 2006; Pardo *et al.*, 2008; Provan *et al.*, 2001; Sánchez-Pérez *et al.*, 2005; Setsuko *et al.*, 2007; Toplin *et al.*, 2008) and for genetic diversity studies in plants such as cedar (Terrab *et al.*, 2006), rice (Ishii and McCouch 2000; Provan *et al.*, 1997), jute (Basu *et al.*, 2004), *Ulex* (Cubas *et al.*, 2005), *Clintonia* (Wang *et al.*, 2011), pine (Powell *et al.*, 1995), potato (Brayan *et al.*, 1999), barley (Provan *et al.*, 1999), soybean (Powell *et al.*, 1996) and kiwi fruit (Weising and Gardner, 1999).

In this study, we used cpSSR markers for the first time to identify a set of polymorphic microsatellite loci and analysis of genetic diversity among and within *P. mahaleb* and *P. avium* species. It is expected that the information of this research will be useful for selection and more efficient utilization of this germplasm in breeding programs in the future.

2. Materials and Methods

2.1 Plant Materials and DNA Extraction

Young leaf samples of 64 genotypes from two *Prunus* species, which include 58 Mahaleb cherry (*P. mahaleb* L.) and six sweet cherry (*P. avium* L.), were used as starting material to carry out a chloroplast microsatellite marker analysis. The genotypes used in this study were obtained from the germplasm collection maintained at the Khorasan Agricultural and Natural Resources Research Centre, Mashhad, and Isfahan University of Technology, Isfahan, Iran. Based on morphological characteristics the plant materials were classified to dwarf and vigorous groups (Table 1). The plant materials were treated with liquid nitrogen and stored at -80°C until being used. Genomic DNA was extracted using the modified cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987). The quality and concentration of the DNA samples were detected on 0.8% agarose gel by electrophoresis and spectrophotometric measurement according to Sambrook and Russell (2001).

2.2 cpSSR Analysis

Twenty five cpSSR primers were selected for cpSSR analysis (Table 2), which purchased from Macrogen Co. (South Korea). PCR reactions were conducted in a 25 μl volume consisting of 2.5 μl of 10x PCR Buffer, 1.5mM MgCl_2 , 1U of *Taq* DNA polymerase, 200 μM of dNTPs, 0.3 μM of each primer and approximately 50 ng of template DNA.

Amplification were carried out in a Peltier Thermal Cycler PTC-0200 (Biorad Co.) with the following PCR program: 5 min of initial denaturing at 94°C , 30 cycles of three steps: 1min of denaturing at 94°C , 1min at appropriate primer annealing temperatures (Table 2), 1min of elongation at 72°C , followed by a final extension of 10 min at 72°C . The PCR products were mixed with 10 μl of formamide loading buffer (95% formamide, 20mM EDTA, pH8.0, 0.25% Xylene cyanol and 0.25% Bromophenol blue) and analyzed on 6% denatured polyacrylamide gels in 1x TBE buffer and then silver stained according to the reported procedure (Bassam *et al.*, 1991; Liu *et al.*, 2007).

2.3 Data Analysis

All clearly detectable and reproducible amplified fragments were scored according to their different allele sizes band and the matrix of cpSSRs data was assembled. The diversity level of gene loci was evaluated with the polymorphic information content (PIC). The PIC value was calculated according to the formula: $PIC_i = 1 - \sum P_{ij}^2$, where P_{ij} is the frequency of the j th allele for the i th marker (Smith *et al.*, 1997).

Distance matrix between genotypes was calculated and a dendrogram was constructed by the genetic distance matrix to display relationships among genotypes using MEGA4 software (Tamura *et al.*, 2007) with Maximum Composite likelihood model and the Neighbor-joining method. Bootstrap analysis with 1000 replicates was also performed to obtain the confidence of branches of the cluster tree.

The chloroplast haplotypes of each individual were generated by combination of alleles detected from the polymorphic primers, because of the non-recombination nature of the chloroplast genome. cpDNA haplotypes were treated as alleles at a single locus. Multilocus haplotypes were generated by combining information from all polymorphic loci. Diversity values based on haplotype frequencies were calculated using Arlequin 3.1 software (Excoffier *et al.*, 2005).

For each cpSSR marker, the presence or absence of each single fragment was coded as 1 or 0, respectively to generate a binary data matrix. Genetic relationships among genotypes were further analyzed by the principal component analysis (PCA) of a similarity matrix according to the extracted Eigen vectors in NTSYS-pc version 2.02i (Rohlf 2000).

Population genetic analysis was performed using the model for co-dominant markers with haploid individuals using POPGENE version 1.32 (Yeh *et al.*, 1999) to calculate observed number of alleles (N_a), effective number of alleles per locus (N_e), Nei's gene diversity (H) (Levene 1949; Nei 1973) and Shannon's information index (I) (Lewontin 1972).

Analysis of molecular variance (AMOVA) was performed to estimate variance components for cpSSR data, partitioning the variation into within and among populations, using Arlequin 3.1 software (Excoffier *et al.*, 2005) with 1000 bootstrap replicates.

3. Results

3.1 Allelic Variation of cpSSR Loci

Twenty five cpSSR primer pairs were used to amplify DNA fragments from 64 *P. mahaleb* and *P. avium* genotypes and 16 out of them were amplified fragments in all genotypes. Eleven out of 16 primer pairs were showed polymorphic bands with a number of alleles ranging from two to four. A total of 25 alleles were identified with an average of 2.27 alleles per locus, while effective number of alleles (N_e) varied from 1.2 to 1.91 with a mean value of 1.56 (Table 3). The genotypes studied revealed significant levels of cpDNA genetic diversity, with percentage of polymorphic bands (PPB) of 68.75%. The mean of polymorphism information content (PIC) was 0.32 which ranged from 0.17 to 0.48 (Table 3). At each single primer pair, the average values of Nei's gene diversity (H) and Shannon's information index (I) were 0.35 (range: 0.17-0.48) and 0.55 (range: 0.31-0.67), respectively (Table 3).

The combination of the alleles at each of the eleven polymorphic loci constituted 43 haplotypes (Table 4). Ten haplotypes (H2, H3, H4, H5, H6, H7, H10, H12, H16 and H36) being found in more than one genotype and the rest of them were in a single genotype. The most of haplotype frequency (0.0862) was seen in five genotypes (T11, T27, T96, T106 and T143) and the lowest one (0.0172) was observed in 29 genotypes (Table 4).

3.2 Genetic Relationships among Genotypes

The distance matrix of the 64 genotypes based on cpSSR analysis constructed by MEGA4 software, showed that genetic relationships of 64 genotypes were different and the range of distance varied from 0.0 to 0.427 with an average of 0.22 (data not shown). The lowest genetic distance was showed between "T143" and "T96" genotypes (0.0), whereas the most genetic distance was between "T263" and "Azadi6" genotypes (0.427).

3.3 Cluster Analysis

The dendrogram constructed from the distance matrix based on Maximum Composite likelihood model and the Neighbor-joining method was grouped the 64 genotype into two main clusters and four groups (Fig. 1). Cluster I is the biggest cluster, comprised of 58 Mahaleb cherry genotypes which divided in the three groups and Cluster II consisted of six sweet cherry accessions that clustered in one group, separately.

The first three principal axes of PCA analysis explained 53.05%, 5.84% and 4.18% of the total variation, respectively. Sum of first three PCAs could be represented most of (63.07%) the total variation in the original dimensions and confirmed the results of cluster analysis.

3.4 Population Genetic Structure of Genotypes

Analysis of molecular variance (AMOVA) was performed to differentiation of dwarf and vigorous genotypes and to estimate the percentage of intra and intergroup genetic variation (Table 5, Analysis 1). Although significant variation was observed among the groups ($F_{st} = 0.06$; $P = 0.0049$), 93.88% of the total variance occurred within groups and 6.12% attributed to among groups. The results showed that the haplotype diversity among vigorous genotypes (0.37) is more than dwarf genotypes (0.24).

Another AMOVA analysis was conducted to estimate the percentage of intra and inter-species genetic variation, which revealed a significant variation among the studied species ($F_{st} = 0.56$; $P < 0.001$). This analysis results showed that 55.72% and 44.28% variation accounted for among and within species, respectively (Table 5, Analysis 2). Haplotype diversity within *P. mahaleb* (0.27) was significantly more than *P. avium* species (0.16).

4. Discussion

4.1 Chloroplast Microsatellite Diversity

Assessment of genetic diversity is an essential component which improved breeding of species. Results obtained in genetic diversity studies of *P. mahaleb* and *P. avium* based on morphological characteristics and molecular markers indicated that abundant genetic diversity exists in these species (Ganji Moghadam and Khalighi 2006; Lacis *et al.*, 2009; Pedersen 2006; Rakonjac *et al.*, 1996 Wunsch and Hormaza 2002 and 2004). Using cpSSR as a basis molecular marker in this study is the first attempt to determine genetic variation among and within *P. mahaleb* and *P. avium* genotypes. The PPB (68.75%) on the species level was near with that of detected using nuclear SSR markers on the Latvian and Swedish sweet cherry (*Prunus avium* L.) (Lacis *et al.*, 2009). The number of alleles per locus in this study (Table 3) was similar with that of pines, *Clintonia* Raf and almond (Echt *et al.*, 1998; Wang *et al.*, 2011; Zeinalabedini *et al.*, 2010; Zhang *et al.*, 2004;) while lower than that of some species (Jiang *et al.*, 2004; Sánchez-Pérez *et al.*, 2005; Setsuko *et al.*, 2007) using nuclear SSR. These show that a lower level of polymorphic for a single locus is detected using cpSSR than using nuclear SSR.

The mean of PIC in this study was in the range of 0.25 to 0.5 ($0.5 > \text{PIC} > 0.25$). This indicates that the cpSSR markers could develop medium loci polymorphism which is useful for genetic variation of genotypes studied (Vaiman *et al.*, 1994; Xie *et al.*, 2010).

Average heterozygosity or gene diversity (H_e) is more appropriate than the proportion of polymorphic loci in assessment of genetic variation (Nei 1987). The mean of heterozygosity calculated for each primer pair in this study was similar with that sweet cherries in other study (Schueler *et al.*, 2003; Wunsch and Hormaza 2004), peach (Sosinski *et al.*, 2000; Testolin *et al.*, 2000) and apricot (Hormaza 2002). The mean of Shannon's information index in this research was 0.55; in agreement with the results of Jin *et al.*, (2008), Xie *et al.*, (2010) and Xu-Xiao *et al.*, (2008). Shannon's information index (Lewontin 1972) was calculated to provide a relative estimate of the degree of variation within genotypes.

4.2 Cluster Analysis and Population Genetic Structure

The clustering results based on polymorphic cpSSR loci fit well to the genetic distance matrix. It was noticed according to dendrogram that *P. mahaleb* L. and *P. avium* L. species were separated which shows the ability of cpSSR markers to separate these two species. Results of clustering showed that "T204" genotype from *P. mahaleb* L. as having the closest genetic relationship with sweet cherry accessions supporting the hypothesis which was based mainly on morphological characteristics. Also, this suggested that "T204" probably arose by hybridization with *P. mahaleb* L. and *P. avium* L.

Some aspects of interrelation among materials studied that were not recognizable by cluster, revealed by the principal components analysis (PCA). Sum of first three PCAs in this study were 63.07%, which this result demonstrates proper distribution of cpSSR markers through entire genome and confirmed the results of cluster analysis; in agreement with the results of Wang *et al.*, (2011).

The large amount of variation attributed to differences within groups (93.88%) and F_{st} value (0.06) showed by AMOVA analysis of two groups of the genotypes which shows a moderate differentiation. In another result of this analysis the differences among species showed the most of variation (55.72%) and the rest was attributed to differences within species and F_{st} value was 0.56. F_{st} value above 0.25 indicated high genetic variation (Wright 1978), and gene flow was limited among the species. Based on these results, the allele numbers among groups

and species studied in this research were more than those observed within them, which shows strong differentiation. These results are in agreement with the results of Wang *et al.*, (2008) and Zhao *et al.*, (2010).

5. Conclusion

In conclusion, chloroplast microsatellite primers used in this study were able to separate genotypes and species of *P. mahaleb* L. and *P. avium* L. according to determine genetic diversity among them. Therefore, cpSSR markers provided a good tool for assessment of genetic diversity among and within species. Consequently, an advantage of microsatellites in the study of conservation genetics is the fact that primers developed for one species are frequently applicable to related taxa.

References

- Basu, A., Ghosh, M., Meyer, R., Powell, W., Basak, S.L. & Sen, S. K. (2004). Analysis of genetic diversity in cultivated Jute determined by means of SSR markers and AFLP profiling. *Crop. Sci.*, *44*, 678–685.
- Bassam, B. J., Caetano-anolles, G. & Gresshoff, P. M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.*, *196*, 80–83.
- Bryan, G. J., McNicoll, J., Ramsay, G., Meyer, R. C. & Jong, W. S. D. (1999). Polymorphic simple sequence repeat markers in chloroplast genomes of Solanaceous plants. *Theor. Appl. Genet.*, *99*, 859–867. <http://dx.doi.org/10.1007/s001220051306>
- Casas, A. M., Igartua, E., Balaguer, G. & Moreno, M. A. (1999). Genetic diversity of *Prunus* rootstocks analyzed by RAPD markers. *Euphytica*, *110*, 139–149.
- Cheng, Y. J., Meng, H. J., Guo, W. W. & Deng, X. X. (2006). Universal chloroplast primer pairs for simple sequence repeat analysis in diverse genera of fruit crops. *J. Hortic. Sci. Biotechnol.*, *81*, 132–138.
- Crane, M. B. & Lawrence, W. J. C. (1929). Genetical and cytological aspects of incompatibility and sterility in cultivated fruits. *J. Pomol. Hort. Sci.*, *7*, 276–301.
- Cubas, P., Pardo, C. & Tahiri, H. (2005). Genetic variation and relationships among *Ulex* (Fabaceae) species in southern Spain and northern Morocco assessed by chloroplast microsatellite (cpSSR) markers. *Amer. J. Bot.*, *92*, 2031–2043.
- Dai, W. J., Tang, S. Q. & Liu, Y. H. (2006). Study on genetic diversity of the endangered plant *Abies ziyuanensis* by using chloroplast SSR marker. *Guangxi. Sci.*, *13*, 151–155.
- Doyle, J. J. & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.*, *19*, 11–15.
- Duminil, J. & Di-Michele, M. (2009). Plant species delimitation: a comparison of morphological and molecular markers. *Plant Biosyst.*, *143*, 528–542.
- Echt, C. S., Devernol, L., Anzidemi, & Vendraming, G. (1998). Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Mol. Ecol.*, *7*, 307–317.
- Ercisli, S., Agar, G., Orhan, E. & Yildirim, N. (2007). Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punica granatum* L.) growing in Southern Anatolia Region in Turkey. *Biochem. Syst. Ecol.*, *35*, 764–769. <http://dx.doi.org/10.1016/j.bse.2007.05.014>.
- Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinfo. Online*, *1*, 47–50.
- Ganji Moghadam, E. & Khalighi, A. (2006). Relationship between vigor of Iranian *Prunus mahaleb* L. selected dwarf rootstocks and some morphological characters. *Sci. Hort.*, *111*, 209–212. <http://dx.doi.org/10.1016/j.scienta.2006.10.028>
- Hormaza, J. I. (2002). Molecular characterisation and similarity relationships among apricot (*Prunus armeniaca* L.) genotypes using simple sequence repeats. *Theor. Appl. Genet.*, *104*, 321–328.
- Ishii, T. & McCouch, S. R. (2000). Microsatellites and microsynteny in the chloroplast genomes of *Oryza* and eight other Gramineae species. *Theor. Appl. Genet.*, *100*, 1257–1266. <http://dx.doi.org/10.1007/s001220051432>
- Jiang, C. S., Ma, X. R., Zou, D. M. & Zhang, Y. Z. (2004). SSR analysis of genetic diversity among *Stylosanthes guianensis* accessions. *High. Tech. Lett.*, *14*, 238–241.
- Jin, L., Jian-Ping, G., Dong-Xu, X., Xiao-Yan, Z., Jing, G. & Xu-Xiao, Z. (2008). SSR analysis of genetic diversity among *Stylosanthes guianensis* accessions. *Acta. Agron. Sin.*, *34*, 1901–1909.

- Jakobek, L., Seruga, M., Voca, S., Sindrak, Z. & Dobricevic, N. (2009). Flavonol and phenolic acid composition of sweet cherries (cv. Lapins) produced on six different vegetative rootstocks. *Sci. Hort.*, 123, 23–28. <http://dx.doi.org/10.1016/j.scienta.2009.07.012>
- Lacis, G., Rashal, I., Ruisa, S., Trajkovski, V. & Iezzoni, A. (2009). Assessment of genetic diversity of Latvian and Swedish sweet cherry (*Prunus avium* L.) genetic resources collections by using SSR (microsatellite) markers. *Sci. Hort.*, 121, 451–457. <http://dx.doi.org/10.1016/j.scienta.2009.03.016>
- Levene, H. (1949). On a matching problem in genetics. *Ann. Math. Stat.*, 20, 91–94. <http://dx.doi.org/10.1214/aoms/1177730093>
- Lewontin, R.C. (1972). The apportionment of human diversity. *Evol. Biol.*, 6, 381–398.
- Liu, L., Zhu, X., Gong, Y., Song, X., Wang, Y., Zhao, L. & Wang, L. (2007). Genetic diversity analysis of radish germplasm with RAPD, AFLP and SRAP markers. *Acta Hort.*, 760, 125–130.
- Malay, C.S., Carolyn, A.Y. & Andrew, A. H. (2009). Genetic variation within and among wild rye (*Elymus canadensis* and *E. virginicus*) populations from the Southern Great Plains. *Crop. Sci.*, 49, 913–922. <http://dx.doi.org/10.2135/cropsci2008.04.0239>
- Mehes, M., Nkongolo, K. K. & Michael, P. (2009). Assessing genetic diversity and structure of fragmented populations of eastern white pine (*Pinus strobus*) and western white pine (*P. monticola*) for conservation management. *J. Plant. Ecol.*, 2, 143–151. <http://dx.doi.org/10.1093/jpe/rtp016>
- Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA*.
- Nei, M. (1987). Molecular evolutionary genetics. Columbia University Press, New York.
- Pardo, C., Cubas, P. & Tahiri, H. (2008) Genetic variation and phylogeography of *Stauracanthus* (Fabaceae, Genisteae) from the Iberian Peninsula and northern Morocco assessed by chloroplast microsatellite (cpSSR) markers. *Amer. J. Bot.*, 95, 98–109.
- Pedersen, B. H. (2006). DNA fingerprints of 51 sweet and sour *Prunus* accessions using simple sequence repeats. *J. Hort. Sci. Biotech.*, 81, 118–124.
- Powell, W., Morgante, M. & Andre, C. (1995). Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. *Curr. Biol.*, 5, 1023–1029.
- Powell, W., Morgante, M., Doyle, J. J., Mcnicol, J. W., Tingey, S. V. & Rafalski, A. J. (1996). Genepool variation in genus *Glycine* subgenus *Soja* revealed by polymorphic nuclear and chloroplast microsatellites. *Genetics*, 144, 793–803.
- Provan, J., Powell, W. & Hollingsworth, P. M. (2001). Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends. Ecol. Evol.*, 16, 142–147.
- Provan, J., Russell, J. R., Booth, A. & Powell, W. (1999). Polymorphic chloroplast simple sequence repeat primers for systematic and population studied in the genus *Hordeum*. *Mol. Ecol.*, 8, 505–511.
- Provan, J., Wilson, N. J. & Soranzo, N. (1998). The use uniparentally inherited simple sequence repeat markers in plant population studies and systematics. In: Molecular Systematics and Plant Evolution (eds Hollingsworth M, Gornall R, Bateman R). Taylor and Frac. London. In press.
- Rakonjac, V., Surlan-Momiravac, G. & Ljubanovic-Ralevic, I. (1996). Morphological and biochemical variability in different populations of wild sweet cherry. *Acta Hort.*, 410, 413–421.
- Rohlf, F. J. (2000). NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software, New York.
- Sambrook, J. & Russell, D. (2001). Molecular cloning, A laboratory manual, Cold Spring Harbor Laboratory Press, New York.
- Sánchez-Pérez, R., Ruiz, D., Dicenta, F., Egea, J. & Martinez-Gomez, P. (2005). Application of simple sequence repeat (SSR) markers in apricot breeding: molecular characterization, protection, and genetic relationships. *Sci. Hort.*, 103, 305–315. <http://dx.doi.org/10.1016/j.scienta.2004.06.009>
- Schueler, S., Tusch, A., Schuster, M. & Ziegenhangen, B. (2003). Characterization of microsatellites in wild and sweet cherry (*Prunus avium* L.) – markers for individual identification and reproductive processes. *Genome*, 46, 95–102.

- Setsuko, S., Ishida, K., Ueno, S., Tsumura, Y. & Tomaru N. (2007). Population differentiation and gene flow within a metapopulation of a threatened tree, *Magnolia stellata* (Magnoliaceae). *Amer. J. Bot.*, *94*, 128–136.
- Smith, J. S. C., Chin, E. C. L., Shu, H., Smith, O. S., Wall, S. J., Senior, M. L., Mitchell, S. E., Kresovich, S. & Ziegler, J. (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLP and pedigree. *Theor. Appl. Genet.*, *95*, 163–173.
- Sosinski, B., Gannavarapu, M., Hager, L. D., Beck, L. E., King, G. J., Ryder, C. D., Rajapakse, S., Baird, W. V., Ballard, R. E. & Abbott, A. G. (2000). Characterisation of microsatellite markers in peach [*Prunus persica* (L.) Batsch]. *Theor. Appl. Genet.*, *101*, 421–428. <http://dx.doi.org/10.1007/s001220051499>
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, *24*, 1596–1599.
- Tehrani, G. & Brown, S. K. (1992). Pollen-incompatibility and self fertility in sweet cherry. *Plant Breed. Rev.*, *9*, 368–370. <http://dx.doi.org/10.1002/9780470650363.ch10>
- Terrab, A., Paun, O., Talavera, S., Tremetsberger, K., Arista, M. & Stuessy, T. F. (2006). Genetic diversity and population structure in natural populations of Moroccan Atlas cedar (*Cedrus atlantica*; Pinaceae) determined with cpSSR markers. *Amer. J. Bot.*, *93*, 1274–1280.
- Testolin, R., Marrazzo, T., Cipriani, G., Quarta, R., Verde, I., Cettori, M. T., Pancaldi, M. & Sansavini, S. (2000). Microsatellite DNA in peach (*Prunus persica* L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. *Genome*, *43*, 512–520.
- Toplin, J. A., Norris, T. B., Lehr, C. R., McDermott, T. R. & Castenholz, R. W. (2008). Biogeographic and phylogenetic diversity of *Thermoacidophilic cyanidiales* in Yellowstone National Park, Japan, and New Zealand. *Appl. Environ. Microbiol.*, *7*, 2822–2833.
- Turkoglu, Z., Bilgener, S., Ercisli, S., Bakir, M., Koc, A., Akbulut, M., Gercekcioglu, R., Gunes, M. & Esitken, A. (2010). Simple sequence repeat-based assessment of genetic relationships among *Prunus* rootstocks. *Genet. Mol. Res.*, *9*, 2156–2165. <http://dx.doi.org/10.4238/vol9-4gmr957>
- Vaiman, D., Mercier, D. & Moazai, G. (1994). A set of 99 cattle microsatellite, characterization, synteny mapping and polymorphism. *Mamm. Genome*, *5*, 288–297.
- Wang, X., Zhao, F., Hu, Z., Critchley, A., Morrell, S. & Duan, D. (2008). Inter-simple sequence repeat (ISSR) analysis of genetic variation of *Chondrus crispus* populations from North Atlantic. *Aquat. Bot.*, *88*, 154–159. <http://dx.doi.org/10.1016/j.aquabot.2007.10.001>
- Wang, Y., Guo, J. & Zhao, G. (2011). Chloroplast microsatellite diversity of *Clintonia udensis* (Liliaceae) populations in East Asia. *Biochem. Syst. Ecol.*, *39*, 22–30. <http://dx.doi.org/10.1016/j.bse.2010.12.015>
- Weising, K. & Gardner, R. C. (1999). A set of conserved PCR primers for analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome*, *42*, 9–19.
- Wright, S. (1978). Evolution and the genetics of populations. University of Chicago Press, Chicago.
- Wunsch, A. & Hormaza, J. I. (2002). Molecular characterisation of sweet cherry (*Prunus avium* L.) genotypes using peach [*Prunus persica* (L.) Batsch] SSR sequences. *Heredity*, *89*, 56–63.
- Wunsch, A. & Hormaza, J. I. (2004). Molecular evaluation of genetic diversity and S-allele composition of local Spanish sweet cherry (*Prunus avium* L.) cultivars. *Genet. Res. Crop. Evol.*, *51*, 635–641. <http://dx.doi.org/10.1038/sj.hdy.6800101>
- Xie, W., Zhang, X., Cai, H., Liu, W. & Peng, Y. (2010). Genetic diversity analysis and transferability of cereal EST-SSR markers to orchardgrass (*Dactylis glomerata* L.). *Biochem. Syst. Ecol.*, *38*, 740–749. <http://dx.doi.org/10.1016/j.bse.2010.06.009>
- Xu-Xiao, Z., Jian-Ping, G., Shu-Min, W. & Qing-Chang, L. (2008). Genetic diversity among Chinese Pea (*Pisum sativum* L.) landraces as revealed by SSR Markers. *Acta. Agron. Sin.*, *34*, 1330–1338. <http://dx.doi.org/10.3724/SP.J.1006.2008.01330>
- Yan, J., Chu, H. J., Wang, H. C., Li, J. Q. & Sang, T. (2009). Population genetic structure of two *Medicago* species shaped by distinct life form, mating system and seed dispersal. *Ann. Bot.*, *103*, 825–834. <http://dx.doi.org/10.1093/aob/mcp006>

Yeh, F. C., Yang, R. C., Boyle, T., Ye, Z. H. & Mao, J. X. (1999). POPGENE, the user friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton, Canada.

Zamani, Z., Sarkhosh, A., Fatahi, R. & Ebadi, A. (2007). Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. *J. Hort. Sci. Biotech.*, 82, 11-18.

Zeinalabedini, M., Khayam-Nekoui, M., Grigorian, V., Gradziel, T. M. & Martinez-Gomez, P. (2010). The origin and dissemination of the cultivated almond as determined by nuclear and chloroplast SSR marker analysis. *Sci. Hort.*, 125, 593-601. <http://dx.doi.org/10.1016/j.scienta.2010.05.007>.

Zhang, X. Y., Bai, S. J. & Huang, M. R. (2004). Analysis of genetic structure in population of *Larix keampferi* by chloroplast SSR markers. *Heredity*, 26, 486-490.

Zhao, W., Chung, J., Cho, Y., Rha, W., Lee, G., Ma, K., Han, S., Bang, K., Park, C., Kim, S. & Park, Y. (2010). Molecular genetic diversity and population structure in *Lycium* accessions using SSR markers. *C. R. Biologies*, 333, 793-800. <http://dx.doi.org/10.1016/j.crv.2010.10.002>

Table 1. The list of genotypes evaluated in this study with the species and group name

No.	Genotype	Species	Group name	No.	Genotype	Species	Group name
1	T90	<i>P.mahaleb</i>	Dwarf	33	T224	<i>P.mahaleb</i>	Vigorous
2	T85	<i>P.mahaleb</i>	Dwarf	34	T266	<i>P.mahaleb</i>	Vigorous
3	T96	<i>P.mahaleb</i>	Dwarf	35	T241	<i>P.mahaleb</i>	Vigorous
4	T247	<i>P.mahaleb</i>	Dwarf	36	T199	<i>P.mahaleb</i>	Vigorous
5	T184	<i>P.mahaleb</i>	Dwarf	37	T52	<i>P.mahaleb</i>	Vigorous
6	T200	<i>P.mahaleb</i>	Dwarf	38	T6	<i>P.mahaleb</i>	Vigorous
7	T268	<i>P.mahaleb</i>	Dwarf	39	T62	<i>P.mahaleb</i>	Vigorous
8	T165	<i>P.mahaleb</i>	Dwarf	40	T136	<i>P.mahaleb</i>	Vigorous
9	T103	<i>P.mahaleb</i>	Dwarf	41	T41	<i>P.mahaleb</i>	Vigorous
10	T162	<i>P.mahaleb</i>	Dwarf	42	T149	<i>P.mahaleb</i>	Vigorous
11	T106	<i>P.mahaleb</i>	Dwarf	43	T176	<i>P.mahaleb</i>	Vigorous
12	T139	<i>P.mahaleb</i>	Dwarf	44	T131	<i>P.mahaleb</i>	Vigorous
13	T267	<i>P.mahaleb</i>	Dwarf	45	T27	<i>P.mahaleb</i>	Vigorous
14	T109	<i>P.mahaleb</i>	Dwarf	46	T99	<i>P.mahaleb</i>	Vigorous
15	T143	<i>P.mahaleb</i>	Dwarf	47	C6	<i>P.mahaleb</i>	Vigorous
16	T161	<i>P.mahaleb</i>	Dwarf	48	Azadi1	<i>P.avium</i>	Vigorous
17	T228	<i>P.mahaleb</i>	Dwarf	49	Azadi2	<i>P.avium</i>	Vigorous
18	T188	<i>P.mahaleb</i>	Dwarf	50	Azadi3	<i>P.avium</i>	Vigorous
19	T205	<i>P.mahaleb</i>	Dwarf	51	Azadi4	<i>P.avium</i>	Vigorous
20	T82	<i>P.mahaleb</i>	Dwarf	52	Azadi5	<i>P.avium</i>	Vigorous
21	T270	<i>P.mahaleb</i>	Dwarf	53	Azadi6	<i>P.avium</i>	Vigorous
22	T108	<i>P.mahaleb</i>	Dwarf	54	T187	<i>P.mahaleb</i>	Vigorous
23	T120	<i>P.mahaleb</i>	Dwarf	55	T227	<i>P.mahaleb</i>	Vigorous
24	T195	<i>P.mahaleb</i>	Dwarf	56	T260	<i>P.mahaleb</i>	Vigorous
25	T11	<i>P.mahaleb</i>	Dwarf	57	T272	<i>P.mahaleb</i>	Vigorous
26	T121	<i>P.mahaleb</i>	Dwarf	58	T204	<i>P.mahaleb</i>	Vigorous
27	T46	<i>P.mahaleb</i>	Dwarf	59	T258	<i>P.mahaleb</i>	Vigorous
28	T155	<i>P.mahaleb</i>	Dwarf	60	T265	<i>P.mahaleb</i>	Vigorous
29	T24	<i>P.mahaleb</i>	Vigorous	61	GZ	<i>P.mahaleb</i>	Vigorous
30	T263	<i>P.mahaleb</i>	Vigorous	62	GX	<i>P.mahaleb</i>	Vigorous
31	T101	<i>P.mahaleb</i>	Vigorous	63	C10	<i>P.mahaleb</i>	Vigorous
32	T83	<i>P.mahaleb</i>	Vigorous	64	C13	<i>P.mahaleb</i>	Vigorous

Table 2. cpSSR primers sequence used in this study

Code	Repeat	Primer Forward5'→3'	Primer Reverse5'→3'	T _m (°C)	Expected size(bp)	Reference
9 NTCP	T ₁₀	CTTCCAAGCTAACGATGC	CTGTCCTATCCATTAGACAATG	55	237	Bryan et al. (1999)
NTCP12	T ₁₀	CCTCCATCATCTCTTCCAA	ATTTATTTTCAGTTCAGGGTTCC	60	136	Bryan et al. (1999)
NTCP18	T ₁₀	CTGTTCTTTCCATGACCCCTC	CCACCTAGCCAAGCCAGA	60	186	Bryan et al. (1999)
NTCP40	A ₁₄	GATGTAGCCAAGTGATCA	TAATTTGATTCTTCGTCGC	55	163	Bryan et al. (1999)
Re3	A ₁₀	TAGGCATAATTCCCAACCCA	CTTATCCATTGGAGCATAGGG	55	129	Ishii and McCouch (2000)
Re5	T ₁₀	ATTTGGAATTTGGACATTTGG	ACTGATTCGTAGGCGTGGAC	55	143	Ishii and McCouch (2000)
Re6	A ₁₀	GAATTTTAGAACTTTGAATTTTACCC	AAGCGTACCGAAGACTCGAA	55	111	Ishii and McCouch (2000)
Re9	T ₁₀	ATAAGGTTATTTCCCGCTTACC	AAATTGGGGGAATTCGTACC	55	144	Ishii and McCouch (2000)
ARCP1	A ₁₅	GAACGACGGGAATTGAACC	GGTGGAATTTGCTACCTTTTT	55	163	Cheng at al. (2006)
ARCP2	A ₁₃	TGGAGAAGGTTCTTTTCAAGC	CGAACCCTCGGTACGATTAA	55	138	Cheng at al. (2006)
ARCP4	T ₁₇	CAATTCGGGATTTTCCTTGA	GAGCGAAGGGGTACGAAATA	59	237	Cheng at al. (2006)
ARCP5	T ₁₃	GGCCATAGGCTGGAAAGTCT	GTTTATGCATGGCGAAAAGG	60	212	Cheng at al. (2006)
ARCP7	A ₈	TTTACCGAGCAGGTCTACG	TGAACGATCCCCAGGACTTA	55	199	Cheng at al. (2006)
ARCP9	A ₁₀ GA ₁₀	GAAAAATGCAAGCACGGTTT	TACGATCCGTAGTGGGTTGC	55	124	Cheng at al. (2006)
ARCP11	A ₁₇	GAGCGAAGGGGTACGAAATA	CAATTCGGGATTTTCCTTGA	59	237	Cheng at al. (2006)
ccmp1	T ₁₀	CAGGTAACTTCTCAACGGA	CCGAAGTCAAAGAGCGATT	52	139	Weising and Gardner (1999)
ccmp2	(A) ₁₁	GATCCCGACGTAATCCTG	ATCGTACCGAGGGTTCGAAT	53	189	Weising and Gardner (1999)
ccmp3	(T) ₁₁	CAGACCAAAGCTGACATAG	GTTTCATTGCTCCTTTAT	48	112	Weising and Gardner (1999)
ccmp4	T ₁₃	AATGCTGAATCGAYGACCTA	CCAAAATATTBGGAGGACTCT	50	126	Weising and Gardner (1999)
ccmp5	(C) ₇ (T) ₁₀ (T) ₅ C(A) ₁₁	TGTTCCAATATCTTCTTGTCATT	AGGTTCCATCGGAACAATTAT	51	121	Weising and Gardner (1999)
ccmp6	(T) ₅ C(T) ₁₇	CGATGCATATGTAGAAAGCC	CATTACGTGCGACTATCTCC	49	103	Weising and Gardner (1999)
ccmp7	(A) ₁₃	CAACATATACCACTGTCAAG	ACATCATTATTGTATACTCTTTC	42	133	Weising and Gardner (1999)
ccmp8	(T) ₆ C(T) ₁₄	TTGGCTACTCTAACCTTCCC	TTCTTTCTTATTTCGCAGDGAA	51	77	Weising and Gardner (1999)
ccmp9	(T) ₁₁	GGATTTGTACATATAGGACA	CTCAACTCTAAGAAATACTTG	41	98	Weising and Gardner (1999)
ccmp10	(T) ₁₄	TTTTTTTTTAGTGAACGTGTCA	TTCGTCGDCGTAGTAAATAG	48	103	Weising and Gardner (1999)

Table 3. Number of the allele, major allele frequency, Gene Diversity, PIC, Nei's gene diversity and Shannon's information index (*I*) for each cpSSR primers

Locus name	Na ^a	Ne ^b	H ^c	I ^d	PIC
ccmp2	4	1.65	0.39	0.58	0.2
ccmp3	2	1.60	0.37	0.56	0.38
ccmp4	2	1.20	0.17	0.31	0.17
ccmp7	2	1.72	0.42	0.61	0.42
AP4	2	1.68	0.40	0.59	0.4
AP5	2	1.64	0.39	0.58	0.38
AP7	2	1.91	0.48	0.67	0.48
AP11	2	1.75	0.43	0.62	0.43
NP9	3	1.47	0.32	0.60	0.21
NP40	2	1.32	0.24	0.41	0.24
NP18	2	1.24	0.19	0.35	0.19
Mean	2.27	1.56	0.35	0.55	0.32

^a Na = Observed number of alleles^b Ne = Effective number of alleles^c H = Nei's (1973) gene diversity^d I = Shannon's Information index

Table 4. Haplotype detected in the genotypes by cpSSR analysis

Haplotype	Genotype name	Haplotype frequencies	Haplotype	Genotype name	Haplotype frequencies
H1	T6	0.0172	H15	T121	0.0172
H2	T11	0.0862	H16	T165	0.0517
	T27			T228	
	T96			T241	
	T106		H17	T176	0.0172
	T143		H18	T187	0.0172
H3	T24	0.0517	H19	T184	0.0172
	T46		H20	T188	0.0172
	T149		H21	T195	0.0172
H4	T41	0.0345	H22	T200	0.0172
	T155		H23	T205	0.0172
H5	T52	0.069	H24	T224	0.0172
	T139		H25	T227	0.0172
	T161		H26	T260	0.0172
	T247		H27	T266	0.0172
H6	T62	0.069	H28	T267	0.0172
	T83		H29	T268	0.0172
	T263		H30	T270	0.0172
	T272		H31	GZ	0.0172
H7	T82	0.0517	H32	C6	0.0172
	T99		H33	C10	0.0172
	T199		H34	C13	0.0172
H8	T85	0.0172	H35	GX	0.0172
H9	T90	0.0172	H36	Azadi1	0.333
H10	T101	0.0517		Azadi2	
	T131		H37	Azadi3	0.167
	T136		H38	Azadi4	0.167
H11	T103	0.0172	H39	Azadi5	0.167
H12	T108	0.0345	H40	Azadi6	0.167
	T162		H41	T204	0.0172
H13	T109	0.0172	H42	T258	0.0172
H14	T120	0.0172	H43	T265	0.0172

Table 5. Analysis of molecular variance (AMOVA) in groups and species of studied genotypes

Analysis	Source of variation	Degree of freedom	Sum of squares	Variance components	% of variance	P value	*F _{ST}
1	Among groups	1	5.73	0.12	6.12	0.0049	0.06
	Within groups	62	116.33	1.87	93.88	-	-
2	Among species	1	23.37	2	55.72	< 0.001	0.56
	Within species	62	98.7	1.6	44.28	-	-

*F_{ST} = Fixation Index

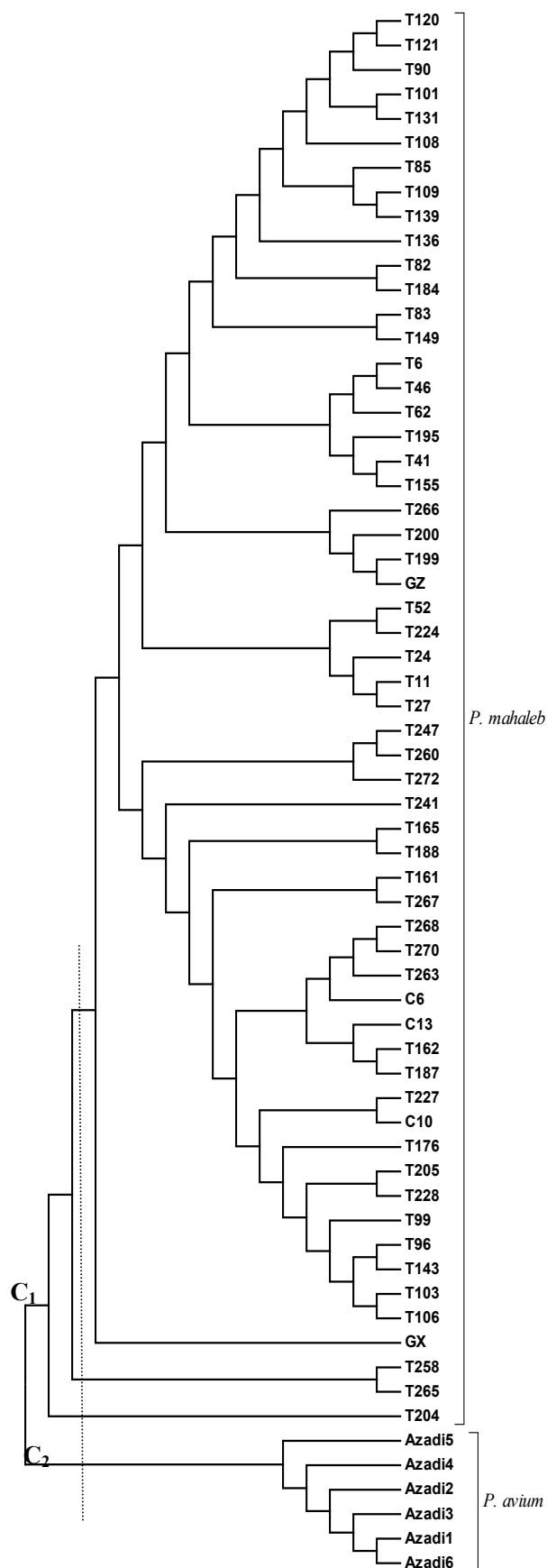


Figure 1. Dendrogram of 64 genotypes, based on cpSSR markers data, by MEGA4 software with Maximum Composite likelihood model and the Neighbor-joining method