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Diversity assessment of wild cherry germplasm by using RAPD markers

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Abstract

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The value of wild relatives of cultivated plants for food safety is widely recognized, but there is still a lack of knowledge about the diversity that exists and how this diversity can be used to improve cultivated plants. The genetic integrity of the wild fruit species is threatened by habitat loss due to increased fragmentation and degradation of the forest and the hybridization with the cultivated varieties. *Prunus avium* L. have been listed on a EUFORGEN priority lists for development of conservation strategies. Investigations of the genetic diversity and structure of local populations are required to determine the most suitable conservation policies for this species at different scales. In this study the RAPD markers were used in order to evaluate genetic similarity between 31 wild cherry trees from 13different test polygons. The test polygons are placed in Forest park Starčevica close to Banja Luka, Bosnia and Herzegovina. Primers OPA-04, OPA-07, OPA-14, OPA-15, OPA-17, and OPG-10 are used to analyse wild cherry trees. High degree of polymorphism is determined between analysed wild cherry trees which imply that in Forest Park Starčevica are present different genotypes of wild cherry.

Keywords: Prunus avium L.; RAPD markers; genetic similarity
Abbreviations: EUFORGEN – European Forest Genetic Resources Programme; RAPD – Random amplification of polymorphic DNA

Introduction

European ex situ collections include a small number of species such as wild relatives. Also, given that these types tend to fall between the priorities of conservation of plant genetic resources for food and agriculture and nature conservation priorities, currently in Europe there is no active conservation of these species either in situ conditions (Maxted & Kell, 2012). Wild fruit species are carriers of genes for resistance to pests, diseases and abiotic stress factors, and as such represent a source of desirable traits in breeding varieties and rootstocks. They are an important component of biodiversity. According to Paunovic et al. (1997) in the region of former Yugoslavia has relatively great number of very important wild fruit species and their relatives, but due to lack of systematic investigations this fact has been practically unknown. Rapid erosion of gene-pool of many species, including wild relatives, poses a serious threat, and the genes that carry the characteristics of adaptation to climate change may be lost forever (Đurić & Mićić, 2015). Researches of ecology and genetic diversity and structure of local populations are required to determine the most suitable conservation policies for this species at different scales (Antic et al., 2017).

Wild cherry (*Prunus avium* L.) belongs to the family *Rosaceae* and is the most important tree species in Europe from this family. Šilić (1964) states that it is frequent in the green belt around Banja Luka. On a European scale, wild cherry is not an endangered species. However, due to its generally scattered and rare occurrence, the genetic diversity of populations can be considered to be under threat from a number of factors such as deforestation, pollution and climate

change. The genetics of sweet cherry has been studied more extensively than wild cherry and since this is the domesticated form of the same species, this information can also be applied to wild cherry (Russell, 2003). The estimation of the diversity of wild fruit populations is crucial for the development of an effective in situ and / or ex situ conservation strategy. Very little information is available on genetic studies for wild species (Namkoong & Koshy, 2001). Preserving genetic resources is vital for future breeding programs and food safety for mankind. In order to conserve genetic resources, objective characterization of individual genotypes of the species is necessary (Karp et al., 1997).

Preservation of the genetic structure of wild cherry (*Prunus avium* L.) and the use of breeding methods are the basis of maintaining its evolutionarily created adaptation potential. Studies of genetic variability and its genetic structure, using molecular markers, have been intensified in most European countries (Tančeva – Crmarić et al., 2011). PCR techniques have been successfully used in the analysis of DNA polymorphism in varieties and cherry pads (*Prunus avium* L.) (Boritzki et al., 2000; Gerlach & Stosser, 1998; Wunsch et al., 2004). A large number of studies have been carried out to determine the genetic relationship between cherry and wild cherry (Marchese et al., 2007, Guarino et al., 2009, Jing-Yong et al., 2009). Although advanced molecular techniques were developed, RAPD is still in use for estimation of genetic variability of different plant species, due to cost and efficiency compared with other methods and a possibility to do RAPD in a moderate laboratory (Danilović et al., 2015; Pinar et al., 2015, Antic et al, 2017). More recently, RAPD markers have been used to study the genetic diversity between 10 autochthonous cherry cultivars in southern Italy (Di Vaio et al., 2015). The ability to identify the variety using one or a few primers indicates that RAPD markers are suitable for safe, easy, fast and inexpensive identification of cherries. These markers are particularly useful for identifying duplicate germplasm in gene banks (Moreno & Trujillo, 2005).

Materials and Methods

The study was conducted in the area of the Starčevica Forest Park close to Banja Luka (Photo 1). The Starčevica Forest Park is surrounded by large settlements and in this forest complex anthropogenic impact is large. The genetic characterization of the studied wild cherries was done using RAPD (Random amplification of polymorphic DNA) molecular markers in order to analyse the genetic diversity of the selected accessions. The number of inventoried locations inside the forest park was 37. Accessions of wild cherry were selected on13 different micro locations (polygons). Each polygon was represented by 1 to 3 trees. In total 31 trees of wild cherry were studied. The genetic diversity was studied among the trees between different polygons and inside the same polygon. In total randomly amplified detection method of DNA (RAPD) is widely used to study the

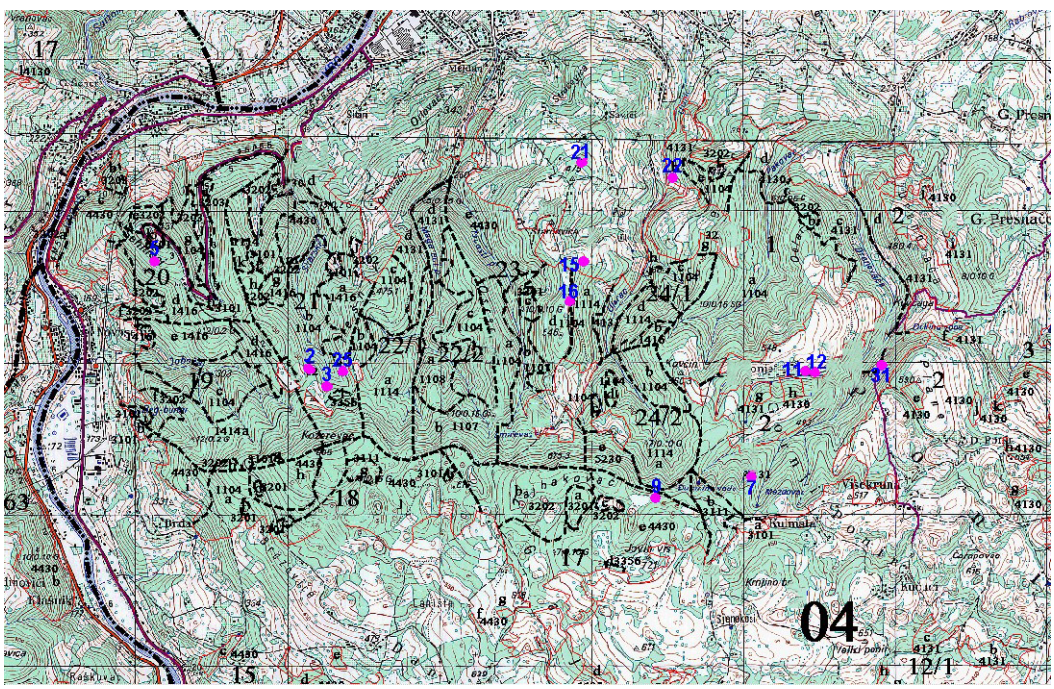


Photo 1. Map of the polygons where cherry accessions were selected

anonymous genomes, such as wild relatives of cultivated species (Dunemann, 1994; Oliviera et al., 1999; Mondini et al., 2009; Lin et al., 2011). The analyses were carried out at the Laboratory of Biotechnology and Molecular Genetics of the Institute of Genetic Resources, University of Banja Luka. The procedure consisted of the following: sampling young leaves; extraction and quantification of DNA; polymerase chain reaction using RAPD primers; separation and visualization of amplified products of agarose electrophoresis and electrophoregrams analysis

Leaf sampling was done by removing a few young leaves from selected trees with a scalpel, wrapped in a moistened paper towel, packed in plastic bags and placed in a hand refrigerator until transported to the laboratory. The leaves samples were stored at 4°C until the next step – DNA isolation. The isolation of the total genomic DNA was performed by CTAB extraction buffer and modified protocol according to Williams et al. (1991). After isolation, the quantification of DNA was done by spectrophotometric measurement of light absorption wavelength of 260 nm. The polymerase chain reaction (PCR) was prepared at a final concentration of 25 µl containing 20 ng DNA, 5 × PCR buffer (Fermentas), 0.2 mM of each of four nucleotides (Fermentas), 3.5 mM MgCl2, 0.5 µM primers, and 0.25 U Taq DNA polymerase (Fermentas). In the experiments six oligonucleotide primers were used in order to establish the molecular analysis of the presence or absence of polymorphism between analysed cherry trees. Commercial names and sequences of the primer are shown in Table 1.

Table 1. Commercial names and sequences of the primer in experiment with wild cherry (Fermentas®)

Primer name	Primer sequence	Concentration
OPA 04	AATCGGGCTG	10 uM
OPA 07	GAAACGGGTG	10 uM
OPA 14	TCTGTGCTGG	10 uM
OPA 15	TTCCGAACCC	10 uM
OPA 17	GACCGCTTGT	10 uM
OPG 20	AGGGCCGTCT	10 uM

The electrophoresis on agarose gel with ethidium bromide is used for the separation and visualization of the polymerase chain reaction products. Electrophoresis was carried out in an electric field voltage of 90 V with a 0.5 × TBE buffer for 60 min. The "wells" on the gel are filled by mixture contained 10 µl of polymerase chain reaction product and 2 µl of loading buffer. The first and last "well" on the gel were filled by the marker Fermentas GeneRuler ™ 100 bp DNA Ladder Plus containing DNA fragments of known lengths from 50-2000 bp. After completion of agarose electrophoresis, the gels were exposed to UV light and photos were taken. Each sample was analysed on the presence or absence of a DNA fragment to a specific length of the primer used. The presence of a specific DNA fragment is described by numerical designation of "1" and the absence by "0". In this way, a numerical matrix is achieved and used to calculate the coefficient of similarity by Jaccard (1908) and to create a dendrogram using SPSS software Version 22.0 (IBM, 2013).

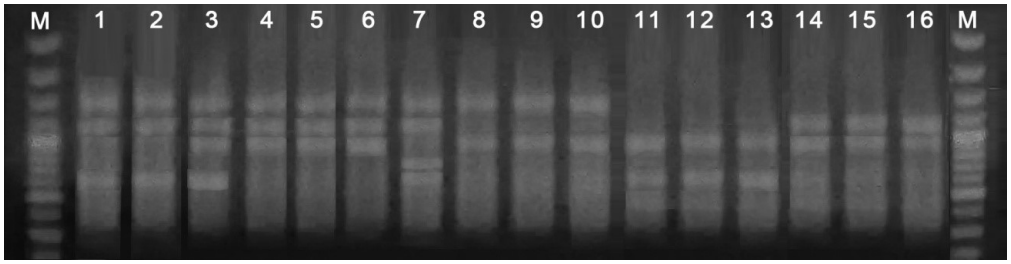
Results

Using the six primers (Table 2) the degree of genetic similarity, between the 31 wild cherry trees inventoried on thirteen polygons, was analysed in the Starčevica forest park. A total of 32 loci were amplified and only one was monomorphic, which represents a high degree of polymorphism. The OPG-20 primers amplified a total of six loci, one of which was monomorphic and the degree of polymorphism was 83%. The OPA-15 and OPA-07 primers amplified six loci and they were all polymorphic and the degree of polymorphism was 100%. The OPA-14 and OPA-04 primers amplified five locuses and were all polymorphic, which means that the degree of polymorphism of the similarity was 100%. The lowest number of amplified loci had primer OPA-17, but highest number of amplified loci has primers OPA-07, OPG-20 and OPA-15. By comparing amplified DNA fragments with fragments of known length (marker GeneRulerTM 100 bp Plus DNA Ladder), the lengths of the fragments expressed in base pairs were determined. Lengths of DNA fragments obtained by polymerase chain reaction with six primers from 31 samples of wild cherry ranged from 350 to 2100 base pairs. An example of RAPD pattern, obtained with the primer OPA-14 is shown in Figure 1 and Figure 2.

Table 2. Number of amplified and polymorphic loci in wild cherry (*Prunus avium* L.)

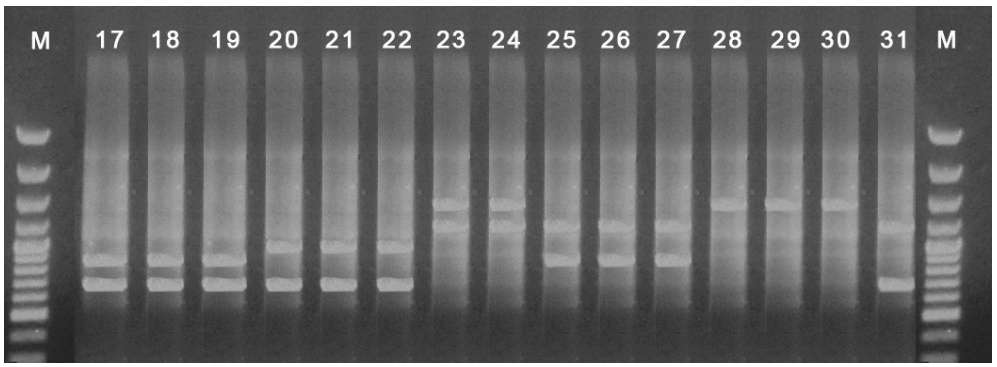
Primer	Sequence	No of amplified loci	Polymorphic loci %
OPA-04	AATCGGGCTG	5	100
OPA-07	GAAACGGGTG	6	100
OPA-14	TCTGTGCTGG	5	100
OPA-15	TTCCGAACCC	6	100
OPA-17	GACCGCTTGT	4	100
OPG-20	AGGGCCGTCT	6	83

The coefficient of genetic similarity was calculated according to Jaccard similarity coefficient (1908) and ranged from 0.04 to 1 among all studied trees. In the 10 polygons, inside the polygons coefficient of the genetic similarity were 1.0, but in the 3 rest polygons the coefficient of similarity



From the left to the right in order: M – marker GeneRulerTM100 bp Plus DNA Ladder, number refers to the accessions studied

Fig. 1. RAPD pattern amplified by primer OPA-14 with 16 samples of wild cherry and comparison with fragments of known length, marker GeneRulerTM100 bp Plus DNA Ladder



From the left to the right in order: M – marker GeneRulerTM100 bp Plus DNA Ladder, number refers to the accessions studied

Fig. 2. RAPD pattern amplified by primer OPA-14 with 15 samples of wild cherry and comparison with fragments of known length, marker GeneRulerTM100 bp Plus DNA Ladder

ranged from 0.80 to 1, which implies that inside the same polygon are found very similar genotypes of wild cherry. The similar results were noticed in research in Slovenia, where is noticed that wild cherries a species that frequently reproduces vegetative, and its natural regeneration could lead to a significant reduction in genetic variability. A visible feature that indicates the vegetative origin of wild cherry is its high density in the stands and short distances between individual trees (Jarni et al., 2012). This also could be explanation for low genetic diversity of wild cherry inside the same test polygon in this research. The coefficient of genetic similarity among polygons ranged from 0.04 to 0.47 which implies that diversity exist among accessions of wild cherry on studied area. The lowest coefficient of genetic similarity is observed between the most distant polygons (P2 and P31). The coefficient of genetic similarity between the polygons that are near shown that genetic similarity between the nearest trees still point significant difference in genetic terms (the highest coefficient of genetic similarity between trees of the nearest polygons was 0.45).

According to the calculated values of Jaccards coefficient, the cluster analyses were done by UPGMA methods comparing all possible pairs. Dendogram was constructed by IBM SPSS Statistic version 22.0 (Figure 3).

This research shows that RAPD markers could be used for identification of wild cherry cultivars in the Starčevica forest park. Using RAPD markers for determine genetic distance between different varieties and accessions of cherry was previously confirmed by other researches (Cai et al., 2006; Zamani et al., 2012; Di Vaio et al., 2015).

Conclusion

The total genetic diversity of wild cherry from the 13polygons in the Starčevica forest park was high. RAPD molecular markers applied in this study determined a high degree of polymorphism among the analysed wild cherry trees. It can be concluded that considerably genetic diversity of wild cherries between different test polygons in the study area is present, i.e. different genotypes of wild cherry are present in the Starčevica Forest Park. When it comes to diversity within the test polygons, we conclude that very similar genotypes of wild cherries generally prevail within a polygon. The obtained results could serve as a basis for the

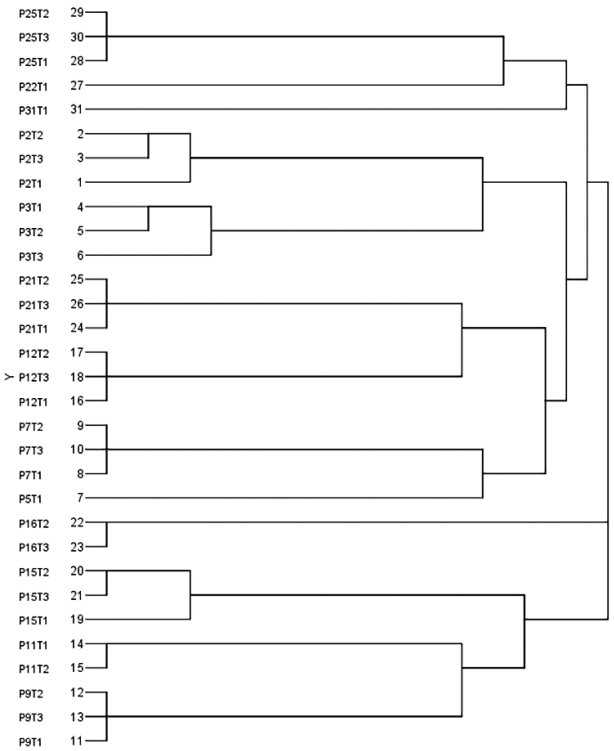


Fig. 3. Dendrogram illustrating the grouping of 31 wild cherry trees (*Prunus avium* L.) obtained by cluster analysis of PCR data using six primers

continuation of the research that needs to be conducted in order to provide strategies and recommendations for the conservation, breeding programmes and use of genetic resources of wild cherry in Bosnia and Herzegovina.

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