Genetic Diversity of Wild Apples and Pears in the Forest Park of Starčevica, Banja Luka, Bosnia and Herzegovina

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Summary

The increasing fragmentation and degradation of forest habitats and the hybridization with cultivated varieties potentially threaten the genetic integrity of wild apple (*Malus sylvestris* /L./ Mill) and wild pear (*Pyrus communis* L.). Wild apple and wild pear have been included in the European Forest Genatic Resources Programme - EUFORGEN priority lists for development of conservation strategies. Researches are required into the genetic diversity and the structure of local populations to determine the most suitable conservation policies for these species at different scales.

In this study, the RAPD markers were used in order to evaluate interspecies genetic similarity of wild apple trees and wild pear trees from the Starčevica Forest Park, Banja Luka, Bosnia and Herzegovina. Primers OPA-05, OPA-07, OPA-09, OPA-10, OPG-03, OPG-11, OPG-12, OPG-13 and OPAC-03 were used to analyse genetic similarity of wild apple trees, while OPA-01, OPA-03, OPA-05, OPA-07, OPA-08, OPD-04, OPD-14, OPG-03 and OPG-06 were used to analyse genetic similarity of wild pear trees. There was a high level of polymorphism among the analysed wild apple trees, as well as among the wild pear trees, demonstrating a considerable richness in terms of wild apple and wild pear genetic resources in the Starčevica Forest Park. The significant genetic diversity of wild apples and wild pears is present between different test polygones, while when it comes to diversity within the test polygons, it can be concluded that very similar genotypes of wild apples and wild pears generally prevail within a polygon.

Key words

Malus sylvestris (L.) Mill, *Pyrus communis* L., RAPD markers, genetic similarity

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Introduction

Wild fruit species are an important component of biodiversity, since they are the carriers of genes for resistance to diseases, pests and abiotic stress factors, and as such represent a source of desirable traits in breeding varieties and rootstocks. According to Paunović et al. (1997), in the region of former Yugoslavia there is a relatively high number of very important wild fruit species and their relatives, but due to a lack of systematic investigations this fact has been practically unknown. Rapid erosion of genepool 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 and Mićić, 2015). Inventory and collection of indigenous germplasm was conducted in Bosnia and Herzegovina, as well as the characterization of indigenous varieties, but inventory and collection of wild species of fruit trees is almost never done (Stevanović et al., 2012)

Wild apple (*Malus sylvestris* /L./ Mill.) and wild pear (*Pyrus communis* L.) are very light-demanding fruit tree species in European forests that are found in a broad range of altitudes and environmental conditions. Genetic variations and structures of both wild apple (*M. silvestris*) and wild pear (*P.. communis*) remain not fully explored and require an extensive research. These two species show significant phenotypic variation and it is assumed that we can distinguish more ecological types due to large areas of natural distribution. Rare and narrow genetic base have caused genetic drift due to a small number of trees and large distances between them. Hybridization with cultivated varieties is also considered to be one of the main obstacles to genetic diversity of wild apples and pears. Crop to-wild introgressions have been reported in the European wild apple *M. sylvestris* that may threaten their genetic integrity (Coart et al., 2003, Larsen et al., 2006, Cornielle et al., 2014). Genetic diversity and hybridization with cultivars were investigated in a population of the endangered European wild apple species *M. sylvestris* with the aim to establish a basis for the implementation of conservation activities in Germany (Reim et al., 2013). According to this study the genetic diversity of *M. sylvestris* population was still high but slightly lower than in the apple cultivars. Reports from Belgium assert that degradation and forest fragmentation have caused a drastic reduction of suitable habitats for wild apple and it is nowadays possible to find only small populations or isolated individuals (Hokanson et al., 1998; Kleinschmit et al., 1998; Keuelmans et al., 2007). According to Katzel (2010), wild pear has been designated as rare and endangered in Germany and should be handled with priority during the conservation of forest genetic resources. Habitat isolation is a consequence of habitat fragmentation that drives a biodiversity loss (Döbert et al., 2014). These small populations with their high genetic diversity represent candidate populations for inclusion in future conservation programmes. 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. In some European countries (Belgium and the Czech Republic), such a research classified *M. sylvestris* as an "endangered species", leading to the dedication of greater resources to *in situ* and *ex situ* conservation programmes (Schnitzler et al., 2014). Diversity assessment of populations of wild fruit trees is of key importance for the development of effective *in situ* and / or *ex situ* conservation strategies. Characterization of diversity has generally long been based on morphological features. However, morphological variations are often influenced by environmental factors. Due to a rapid development in the field of molecular genetics over the last few decades, a lot of different techniques for the analysis of genetic variation appeared (Spooner et al., 2005; Anumalla et al., 2015). Molecular markers avoid many complications of environmental influences and therefore molecular genetics techniques are now used as a complementary strategy to the traditional approach for the characterization of genetic resources (Mataruga et al., 2013). DNA fingerprinting has become an immensely important instrument for genotype identification in both wild plant species and their cultivated relatives (Dunemann, 1994; Mondini et al., 2009; Yadav et al., 2014). The RAPD marker system is easy to apply, since no prior DNA sequence information is needed for designing PCR primers, as required for other PCR-based genetic marker systems (Kovačević et al., 2013). 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). The use of RAPD approaches has shown to be a tool for the differentiation of apple cultivars (Koller et al., 1993; Autio et al., 1998; Oraguzie et al., 2001; Erturk and Akcay, 2010; Kaya et al., 2015). The characterization of the genus *Pyrus* has been carried out by Oliviera et al. (1999) that calculated genetic relationship between cultivars and wild *Pyrus* species. Developed RAPD markers can be used to identify the pear varieties, as well as collected germplasm of this plant (Lisek and Rozpara, 2010). More recently, RAPD were a useful tool for identification of *Pyrus* cultivars in China (Lin et al., 2011) and for identification of autochthonous pear accessions in the Republic of Srpska (Kajkut et al., 2015).

Material and methods

The survey was conducted in the area of the Starčevica Forest Park close to Banja Luka. The Starčevica Forest Park is surrounded by large settlements and in this forest complex anthropogenic impact is large. In total 37 different test polygons (20 x 20 m) were inventoried in order to determine presence of wild apple and pear. Analyses were performed on nine wild apple trees from five different test polygons (P3, P10, P12, P31 and P32) and on 13 wild pear trees from eight different test polygons (P2, P4, P6, P12, P31, P32, P34 and P35). Polygons P10 and P32 were represented by two apple trees and polygon P12 by three apple trees, while polygons P3 and P31 were represented by one apple tree. Polygons P4, P6 and P32 were represented by two pear trees, and polygon P12 by three pear trees, while the polygons P2, P31, P34 and P35 were represented by one pear tree (Table 1). Genetic diversity was evaluated between different polygons and within the same polygon. Genetic characterization was performed using RAPD (Random Amplification of Polymorphic DNA) molecular markers to analyse genetic diversity of selected accessions within individual species. Randomly amplified detection method of DNA (RAPD) is widely used to study the anonymous genomes, such as wild relatives of cultivated species (Dunemann, 1994; Oliviera et al, 1999; Mondini

Table 1. Longitude and latitude of polygons with the number of analysed wild apple and wild pear trees

Polygon	Latitude	Longitude	No. of apple trees analysed	No. of pear trees analysed
P2	44° 44′ 08.9″N	017° 10′ 31.9″E		1
P3	44° 44′ 05.8″N	017° 10′ 37.0″E	1	
P4	44° 44′ 08.5″N	017° 10′ 30.5″E		2
P6	44° 44′ 49.9″N	017° 10′ 26.9″E		2
P10	44° 43′ 25.9″N	017° 12′ 31.4″E	2	
P12	44° 44′ 10.4″N	017° 13′ 05.6″E	3	3
P31	44° 44′ 13.5″N	017° 13′ 27.1″E	1	1
P32	44° 44′ 17.9″N	017° 13′ 27.2″E	2	2
P34	44° 44′ 14.1″N	017° 13′ 08.0″E		1
P35	44° 43′ 21.5″N	017° 13′ 01.0″E		1

et al., 2009; Lin et al., 2011). 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.

Leaves sampling

The sampling of young leaves was carried out during 2014, so that the leaves from selected trees were removed with a scalpel and few leaves were taken from every accession, wrapped in a moist towel, packed in plastic bags and placed in the portable refrigerator until transportation to the laboratory. The leaves were kept in the laboratory at 4°C until the next step and the isolation of DNA.

DNA isolation and PCR

The isolation of total genomic DNA was performed with CTAB extraction buffer and by the 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. An absorbance (A) corresponds to an amount of 50 ng of double-stranded DNA in 1 ml of solution. The polymerase chain reaction (PCR) was prepared at a final concentration of 25 µl. Each reaction contained 20 ng DNA, 5 × PCR buffer (Fermentas), 0.2 mM of each of four nucleotides (Fermentas), 3.5 mM $MgCl_2$, 0.5 µM primers, and 0.25 U Taq DNA polymerase (Fermentas). In the experiments with wild apples and pears nine oligonucleotide primers per experiment were used in order to establish the molecular analysis of the presence or absence of polymorphism between analysed trees. The first stage of the PCR reaction, the activation of the enzyme, was carried out at a temperature of 94°C for a period of five minutes. Amplification of DNA was carried out in 40 cycles at denaturation temperatures of 94°C for 30 seconds and the abutting faces of primers at 37°C for 30 seconds, and elongation, i.e. extending the DNA, at a temperature of 72°C for a period of one minute. After all cycles were completed, prolonged elongation followed for eight minutes at 72°C.

The separation and visualization of the polymerase chain reaction products were carried out by electrophoresis on agarose gel with ethidium bromide. The mixture for filling "wells" on the gel contained 10 µl of polymerase chain reaction product and 2 µl of loading buffer. The first and last "well" on the gel was filled by the marker Fermentas GeneRuler ™ 100 bp DNA Ladder Plus, which contains DNA fragments of known lengths from 50-2000 bp. By using this marker sharply defined bands after agarose electrophoresis were obtained. Electrophoresis was carried out in an electric field voltage of 90 V with a 0.5 × TBE buffer for 60 minutes. After completion of agarose electrophoresis, the gels were exposed to UV light, and then photographing was carried out. 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, we achieved a numerical matrix that is used to calculate the coefficient of similarity by Jaccard (1908) and to create a dendrogram using SPSS software Version 22.0 (IBM, 2013).

Results and discussion

Wild apple (*Malus sylvestris* /L./ Mill.)

The degree of polymorphism was analysed between nine wild apple trees using nine primers where the total of 52 locus was amplified. The percentage of polymorphism generated by primers OPA-05, OPA-09, OPG-03, OPG-11, OPG-12, OPAC-03 was 100%, while the percentage of polymorphism generated by primer OPG-19 was 85%. The lowest level of polymorphism was generated by primers OPA-07 and OPA-10 (83%) (Table 2). As a total these nine primers were polymorphic and generated 94.5% average polymorphism across the nine studied wild apple trees. By comparing the amplified DNA fragments with fragments of known length (GeneRulerTM marker 100 bp DNA Ladder Plus), the length of fragments expressed in base pairs were determined. Lengths of DNA fragments obtained by polymerase chain reaction with nine primers from nine samples of wild apple ranged from 250 to 2000 base pairs.

Table 2. Number of amplified and polymorphic loci in wild apples (*Malus sylvestris* /L./ Mill.)

Primer	Sequence	No. of amplified loci	Percentage of polymorphic loci %
OPA-05	AGGGGTCTTG	6	100
OPA-07	GAAACGGGTG	6	83
OPA-09	GGGTAACGCC	4	100
OPA-10	GTGATCGCAG	6	83
OPG-03	GAGCCCTCCA	5	100
OPG-11	TGCCCGTCGT	6	100
OPG-12	CAGCTCACGA	6	100
OPG-19	GTCAGGGCAA	7	85
OPAC-03	CACTGGCCCA	6	100

The coefficient of genetic similarity by Jaccard was calculated and ranged from 0.20 to 1.00 (Table 3). Wild apples were grouped into five groups according to this coefficient. The first group ranged from 0.20 to 0.24. The second group ranged from 0.31 to 0.35. The third group ranged from 0.40 to 0.48, the fourth group had a coefficient value of 0.54 and the fifth group had a coefficient in the range from 0.83 to 1.

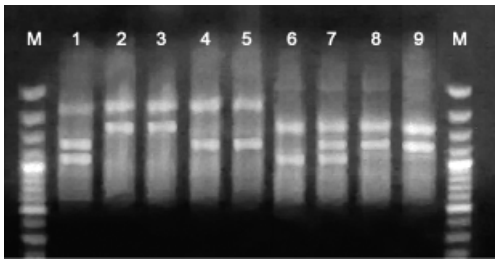

Figure 1. Electrophoregrams obtained by a cyclic reaction of polymerase using the primer OPA 09 with nine samples of wild apple (*Malus sylvestris* /L./ Mill.) and comparison with fragments of known length, marker GeneRulerTM100 bp DNA Ladder Plus (M)

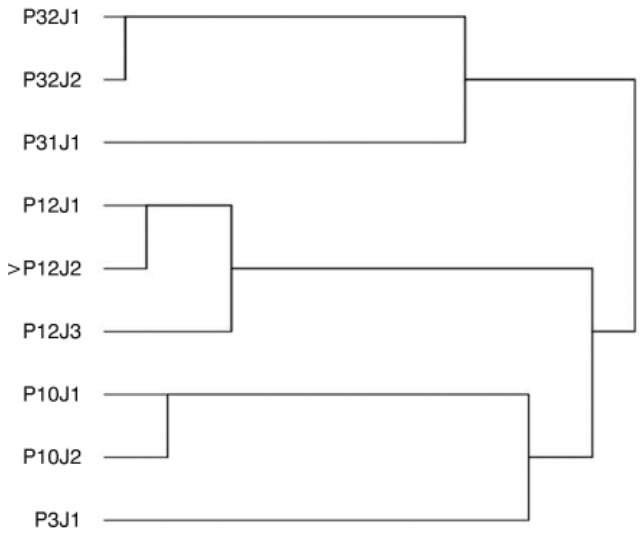

Figure 2. Dendrogram illustrating the grouping of nine wild apple trees (*Malus sylvestris* /L./ Mill.) obtained by cluster analysis of PCR data using nine primers

As shown in Table 3, the coefficient of genetic similarity is the highest inside the test polygons. In test polygon P32 the coefficient value is 1 which means that the trees inside the test polygon P32 most likely belong to the very similar genotype. The coefficient of genetic similarity inside test polygon P10 had the value of 0.92, while inside test polygon P12, its value was in the range from 0.83 to 0.96. The coefficient of genetic similarity between the test polygons was in the range from 0.20 to 0.54, which means that there are most likely diverse genotypes in different polygons. According to the calculated values of Jaccards coefficient, the cluster analyses were done by UPGMA methods comparing all possible pairs. Dendogram is constructed by IBM SPSS Statistic version 22.0 (Fig. 2).

Wild pear (*Pyrus communis* L.)

The degree of polymorphism was analysed between 13 wild pear trees using 9 primers and the total of 45 loci was amplified. The percentage of polymorphism generated by primers OPA-03, OPA-05, OPA-07, OPG-03 and OPD-04 was 100%, while the percentage of polymorphism generated by OPA-01, OPA-08 and OPG-06 was 80%. The lowest degree of polymorphism was generated by primer OPD-14 (50%). As a total these nine primers were polymorphic and generated 87.7% average polymorphism across the 13 studied wild pear trees (Table 4). By comparing amplified DNA fragments with fragments of known length (GeneRulerTM marker 100 bp DNA Ladder Plus), the length of fragments expressed in base pairs was determined. The length of DNA fragments obtained by polymerase chain reaction with nine primers from nine samples of wild pears ranged from 300 to 2000 base pairs (Fig. 3).

The coefficient of genetic similarity by Jaccard was calculated and ranged from 0.26 to 1.00 (Table 5). The pears were grouped into three groups according to the coefficient of genetic similarity. In the first group coefficient varied from 0.26 to 0.39. The second group had a coefficient from 0.45 to 0.58; while the third group had a coefficient from 0.77 to 1.00.

The coefficient of genetic similarity inside of test polygons ranged from 0.87 (P4) to 1.00 (P12) and we can conclude that very similar genotypes are represented inside the test polygons. Also, similar genotypes are noticed in test polygons P2 and P4. The comparison of coefficient of genetic similarity between the remaining of the test polygons showed high difference and diversity. According to the calculated values of Jaccards coefficient, the cluster analyses was done by UPGMA methods comparing all possible pairs. Dendogram was constructed by IBM SPSS Statistic version 22.0. (Fig. 4).

Table 3. Coefficient of similarity between nine wild apple trees (*Malus sylvestris* /L./ Mill.) according to Jaccard

	1:P3J1	2:P10J1	3:P10J2	4:P12J1	5:P12J2	6:P12J3	7:P31J1	8:P32J1	9:P32J2
1:P3J1	1.000								
2:P10J1	0.487	1.000							
3:P10J2	0.436	0.926	1.000						
4:P12J1	0.439	0.310	0.325	1.000					
5:P12J2	0.463	0.333	0.350	0.966	1.000				
6.P12J3	0.463	0.333	0.350	0.839	0.871	1.000			
7:P31J1	0.400	0.486	0.471	0.205	0.200	0.227	1.000		
8:P32J1	0.317	0.351	0.333	0.244	0.238	0.238	0.548	1.000	
9:P32J2	0.317	0.351	0.333	0.244	0.238	0.238	0.548	1.000	1.000

Table 4. Number of amplified and polymorphic loci in wild pears *(Pyrus communis* L.)

Primer	Sequence	No. of amplified loci	Percentage of polymorphic loci %
OPA-01	CAGGCCCTTC	5	80
OPA-03	AGTCAGCCAC	4	100
OPA-05	AGGGGTCTTG	6	100
OPA-07	GAAACGGGTG	5	100
OPA-08	GTGACGTAGG	5	80
OPD-04	TCTGGTGAGG	6	100
OPD-14	CTTCCCCAAG	4	50
OPG-03	GAGCCCTCCA	5	100
OPG–06	GTGCCTAACC	5	80

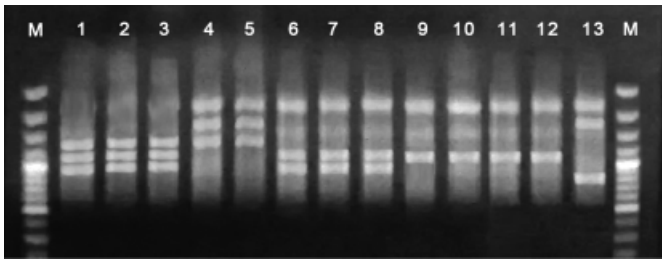

Figure 3. Electrophoregrams obtained by cyclic reaction of polymerase using the primer OPA 07 with 13 samples of wild pears *(Pyrus communis* L.) and comparison with fragments of known length, marker GeneRulerTM100 bp DNA Ladder Plus (M).

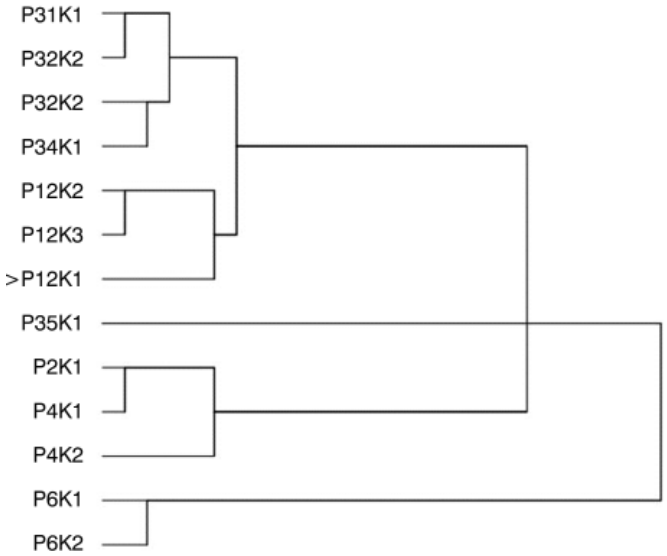

Figure 4. Dendrogram illustrating the grouping of 13 wild pear trees (*Pyrus communis* L.) obtained by cluster analysis of PCR data using nine primers

The high level of polymorphism in our study demonstrated that there is considerable richness in terms of wild apple and wild pear genetic resources in the Starčevica Forest Park. The average plymorphism across the nine studied wild apple trees generated 94.5%. The recent study from Turkey resulted in similar findings, where Kaya et al. (2015) have studied genetic variability and relationships among 35 native apple genotypes using RAPD markers and demonstrated that the level of polymorphism across genotypes was 89.29%. The average polymorphism across the 13 studied wild pear trees generated 87.7%. Oliveira et al. (1999) reported that RAPD techniques revealed high polymorphism percentage (91%) when comparing different pear cultivars derived from different species. Lisek and Rozpara (2010) confirm usefulness of both techniques, RAPD and ISSR in identifying pear cultivars in nurseries and orchards, as well as in germplasm collections of these species. The degree of DNA polymorphism was estimated at 56.3% (RAPD) and 71.5% (ISSR). These studies indicate that RAPD is sufficiently informative and powerful to access genetic variability of natural populations. Even though newer techniques such as AFLP and SSR are favoured due to their informativeness, RAPD is still a method of choice for less advanced laboratories because of its simplicity and low

Table 5. Coefficient of similarity between 13 wild pear trees *(Pyrus communis* L.) according to Jaccard

	1:P2K1	2:P4K1	3:P4K2	4:P6K1	5:P6K2	6:P12K1	7:P12K2	8:P12K3	9:P31K1	10:P32K1	11:P32K2	12:P34K1	13:P35K1
1:P2K1	1.00												
2:P4K1	1.00	1.00											
3:P4K2	0.87	0.87	1.00										
4:P6K1	0.30	0.30	0.27	1.00									
5:P6K2	0.29	0.29	0.26	0.96	1.00								
6:P12K1	0.53	0.53	0.54	0.31	0.33	1.00							
7:P12K2	0.53	0.53	0.55	0.30	0.29	0.88	1.00						
8:P12K3	0.53	0.53	0.55	0.30	0.29	0.88	1.00	1.00					
9:P31K1	0.45	0.45	0.46	0.30	0.30	0.77	0.87	0.87	1.00				
10:P32K1	0.45	0.45	0.46	0.30	0.30	0.77	0.87	0.87	1.00	1.00			
11:P32K2	0.46	0.46	0.48	0.31	0.30	0.80	0.91	0.91	0.95	0.95	1.00		
12:P34K1	0.45	0.45	0.46	0.34	0.33	0.84	0.87	0.87	0.91	0.91	0.95	1.00	
13:P35K1	0.39	0.39	0.45	0.36	0.35	0.58	0.53	0.53	0.45	0.45	0.46	0.50	1.00

cost. Thus, RAPD markers will provide a useful tool in the future design of collection strategies for germplasm conservation (Yadav et al., 2014).

This RAPD analysis showed a high genetic diversity in wild apple and wild pear trees growing in different test polygons and low diversity in trees growing in the same test polygon and in trees growing in the polygons close to one other. Nevertheless, the studied area is close to human settlements and gene flow from the cultivated species to the wild ones is more likely been happening and some of the sampled trees could be natural hybrids. The differentiation between typical representatives of the wild species and their hybrids with cultivated species should be done prior the establishing *in situ* conservation measures. Although the study is severely limited in the number of analysed individuals, it gives a first insight into genetic diversity of the wild apples and wild pears in this area. Having in mind that wild apple and wild pear in the Starčevica Forest Park are found in small populations or as individuals, it is necessary to establish conservation measures in order to protect diversity of wild apple and wild pear in this area.

Conclusion

RAPD molecular markers applied in this study determined a high degree of polymorphism among the analysed wild apple trees, and among wild pear trees. It can be concluded that considerably genetic diversity of wild apples and wild pears between different test polygons in the study area is present, i.e. different genotypes of wild apples and wild pears are present in the Starčevica Forest Park. When it comes to diversity within the test polygons, we conclude that very similar genotypes of wild apples and wild pears generally prevail within a polygon. The diversity of the genetic basis of wild apple and pear, discovered by the results of genetic characterization, presents important guidelines for the restoration and protection of genetic diversity of wild fruit species. Genetic characterization of wild apple and wild pear resources could be helpful in an effective conservation effort of those species, which could be applicable to exchange traits with cultivated varieties in breeding programs.

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