Genetic Variability of Autochthonous Pear Varieties from Tuzla Canton, Bosnia and Herzegovina

Besim Salkić, Ensar Salkić, Amela Hercegovac, Aldijana Avdić, Azra Dorić, and Ahmed Salkić

ABSTRACT

Pear as a fruit species has a special place because of its quality characteristic. The fact that about 20,000 pear seedlings are considered to be sold annually in Bosnia and Herzegovina, which are autochthonous or spontaneously expanded varieties, also speaks in favor. The main goal of this research is to analyze the genetic variability of nine autochthonous pear varieties in Tuzla Canton, Bosnia and Herzegovina to enable the conservation and expansion of existing genetic resources. The study included nine autochthonous pear varieties. Samples of young leaves were collected on the following localities: the town of Srebrenik, the town of Gradačac, the municipality of Čelić-Tuzla Canton, Bosnia and Herzegovina, in the spring 2019. To determine genetic diversity, 12 SSR (Simple Sequence Repeats) markers were genotyped. There were no cases of synonyms or homonyms in the analyzed set. Each of the nine tested varieties represents a unique genotype. Autochthonous pear cultivars analyzed in this paper represent an interesting genetic resource, with useful agronomic traits that can be used in future cultivation.

Keywords: autochthonous varieties, pear, genetic diversity, SSR markers.

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I. INTRODUCTION

The autochthonous gene pool of agricultural plants is invaluable from the point of genotypic structure and in given growing conditions can be a valuable source of genetic variability in selection and breeding.

Characterization of species germplasm is a topic of great interest to many countries that agreed to international standards under the FAO Agreement on Plant Genetic Resources. Genetic identification as well as physico-chemical qualities of fruits of autochthonous pear varieties in Bosnia and Herzegovina represent a scientific research. The trend of planting fruit varieties which are resistant or tolerant to economically significant diseases, as well as customer demands for autochthonous varieties, has led nurseries in Bosnia and Herzegovina to graft and sell a pear assortment

that is not on the Bosnian and Herzegovinas variety list. Grafted autochthonous varieties of pears are of local importance [1].

So, there is a problem of legitimacy of planting material production. The same statement can be applied to pear varieties that are traditionally grown in Bosnia and Herzegovina.

* This is because pears can grow in a wide range of climatic conditions as they are in Bosnia and Herzegovina. Different genotypes ripen from early summer to late autumn, pear fruits can be used in different ways, from fresh consumption to drying, making various processed products such as jams, juices, etc. [2]. Globally, agriculture is developing very fast, and traditional farming methods are being avoided, more and more people are going in the direction of intensive agricultural production based on

modern technologies. The goal is maximizing income per area unit. Growing varieties that are highly productive and resistant to some economic diseases and pests leads to the uniformity of orchards, and thus also reduces genetic variability. Genetic variability is also defined as tendency of individual genotypes in a population to differ from each other. This term does not refer to the essence of the term 'genetic diversity' which means a rate of variation in a population [1]-[4].

Today, there are gene banks in the world that store plant genes, but their capacity is not large, so there are still a large number of plant genes that have not been collected and described.

The variability of a plant is determined by the environmental factors to which the plant adapts. Genetic variability is very important for biodiversity, which tends to adapt to the conditions in which the plant grows. Variability is an important factor which have an effect on individual response of plants to environmental stress and thus can lead to the differential survival of plants in a population under the influence of natural selection. Genetic variability also basically implies the differential susceptibility of organisms to disease and sensitivity.

In the Balkan Peninsula, where life has been accompanied by migrations and wars for centuries, fruit trees are synonymous for life for most people. Fruit trees are a sign that someone has settled in one place and wait for its growth and fruiting. Fruit trees are a sign that people will stay in that place for a long time. We can say that there is an almost cultic attitude towards fruit trees.

This type of attitude towards fruit trees has led to great genetic variability and the possibility of researching fruit trees, which is an extensive job, because they need to be located, identified, and collected.

Pear as a fruit species has a special place because of its quality characteristics. The fact that about 20,000 autochthonous or spontaneously expanded pear seedlings are sold annually in Bosnia and Herzegovina, supports this. The genus *Pyrus* probably originated in Central Asia, the mountainous parts of Western and Southern China, Asia Minor, and India, from where were expanded east and west of its primary center of origin [5]. Diversifications of the genus *Pyrus*, have been observed mainly in East and Central Asia, the Himalayas, the Caucasus, Asia Minor, and Eastern Europe. Maqsood cites research that determines how wild species of the genus *Pyrus* spread from the Balkans, Caucasus, Turkmenistan, Siberia to China, and Japan [6], while some species of the genus are naturalized in the United States, although this continent is not a natural habitat of the genus *Pyrus* [7]. According to Vavilov, they have three centers of origin of cultivated pears: the Chinese center, Central Asian and the Middle Eastern. In addition to these, Zeven and Zhukovsky proposed a fourth, Euro-Siberian center [8]. From the first organized expeditions, the pear was of interest to germplasm collectors. Most cultivars originate from *P. communis* L. [9], [10].

The genetic uniformity of commercial pear varieties in the United States and Europe is extremely high, so pear production is very vulnerable. About 50% of commercial pear production refers to the Williams variety (USDA, 2004). Additionally, there is a high genetic similarity between the

varieties *Williams* and *Conferance*, probably because they originate from close genotypes and share the same genetic pool [11]. The diversity of cultivated plant species generally serves as the basis for biodiversity in agriculture [12]. It is very important to understand that by caring for biological diversity, we are actually caring for millions of lives, food, medicine, traditional and modern pharmacology [13]. Biological diversity is the variability of living organisms in all frameworks-including terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are constituted, and this includes diversity within species, between species and diversity of ecosystems [14].

During the past decades, several researches of autochthonous fruit germplasm in Bosnia and Herzegovina have been done. In a review of the results of previous research conducted in the former Yugoslavia, Paunović *et al.* [19] provided information that this area has 124 registered wild fruit species and relatives. At the same time, research on wild fruits is very important [20], [21]. Paunović *et al.* [19] indicate that the area of BiH can be considered a gene center of some species and their wild relatives: *Malus*, *Pyrus*, *Chaenomeles*, *Sorbus*, *Crataegus*, *Mespilus*, *Eriobotrya*, *Prunus*, *Amygdalus*, *Juglans*, *Corylus*, *Castanea*, *Cornus*, *Morus*, *Sambucus*, *Fragaria*, *Ribes*, *Rubus*, *Rosa*, *Ficus*, *Punica*, *Zizyphus*, *Citrus*.

During the past decades, there have been several studies of autochthonous fruit germplasm in Bosnia and Herzegovina. In a review of the results of previous research conducted in the former Yugoslavia, Paunović and associates [19] provided data that this area has 124 registered wild fruit species. At the same time, the research of wild fruits is very important [20], [21]. Paunović *et al.* point out that the area of Bosnia and Herzegovina can be considered the gene center of some species and their wild relatives from the genera *Malus*, *Pyrus*, *Chaenomeles*, *Sorbus*, *Crataegus*, *Mespilus*, *Eriobotrya*, *Prunus*, *Amygdalus*, *Juglans*, *Corylus*, *Castanea*, *Cornus*, *Morus*, *Sambucus*, *Fragaria*, *Ribes*, *Rubus*, *Rosa*, *Ficus*, *Punica*, *Zizyphus*, *Citrus*. During the seventies of the last century, the area of the Balkans, and thus the former Yugoslavia / Bosnia and Herzegovina, was the subject of a large expedition [22], when a total of 225 varieties of pear were collected in Serbia, Kosovo, Bosnia and Herzegovina, Macedonia and Montenegro. Most of the varieties at the time of sampling were without signs of leaf disease or insect damage, including pear flea. The buds taken for propagation were sent to Glenn Dale (NPKQC-National Plant Germplasm Quarantine Center) where they were propagated on the Bartlett rootstock. The growth was vigorous. There was no effort to prevent infection either by spraying or pruning, unless there was a risk of plant loss. The plants were under observation from 1981-1988. The first big project to collect fruit germplasm, organized by former Yugoslavia researchers, was undertaken during the period 1988 and 1991 through the "Plant Gene Bank of Yugoslavia" project. The project included all activities: inventory, collection, identification, multiplication, characterization, and establishment of a gene bank. However, with the advent of the war years, this work was discontinued, and was resumed after 2000, through the SEED net project - Southeast European Development Network for Plant Genetic Resources [1].

There is more data in the available literature about germplasm characterization of autochthonous apple and pear cultivars [23]-[25] and genetic analysis of homonyms within the autochthonous apple gene pool in Bosnia and Herzegovina using SSR markers [26].

Characterization of germplasm involves activities to determine the expression of properties that are highly conditioned by a hereditary basis, ranging from analysis of morphological characteristics, through analysis of proteins to molecular markers. In Bosnia and Herzegovina, beside the inventory and collection of autochthonous germplasm [1], the characterization of autochthonous apple varieties [29] was also done.

Genetic diversity is essential for the effective management and use of genetic resources. Determination of genetic diversity is possible based on phenotypic/morphological traits or biochemical techniques based on analysis of enzymes, proteins or using DNA analysis based on molecular techniques, such as SSR marker analysis.

Molecular genetic methods are based on the analysis of the variability of hereditary material (DNA), which is very conservative. Hereditary material is transmitted from generation to generation within normal growing conditions through vegetative propagation, as a rule, without the influence of environmental factors. In other words, the content and structure of hereditary material (genomic and cytoplasmic DNA) of new plants during vegetative reproduction usually remain the same - unchanged. Therefore, methods based on the analysis of DNA structure are very suitable for identification purposes, because once determined the characteristics of DNA by the same method in one place can be checked by the same method in any other place and at any stage of plant development. Variation of weather conditions, soil type or altitude can have a significant impact on the phenotype characteristics of variety, while the genetic profile determined by molecular genetic analysis even under changed conditions will remain unchanged. If the established genetic profile of a variety differs from the profile of other varieties, then its stable repeatability can be successfully used for identifying of varieties, which is crucial in the characterization of germplasm in fruit growing and viticulture [30]. The main goal of this research is to analyze genetic variability of nine autochthonous pear varieties in Tuzla Canton, Bosnia and Herzegovina in order to preserve and expand existing genetic resources through registered nurseries.

II. MATERIAL AND METHODS

A. Sampling

The research included nine autochthonous pear varieties from Bosnia and Herzegovina, as follows: Mesnjača, Žutica, Ječmenka, Pšeničnjača, Savka, Okrugla, Karamanka, Huremka i Kongreska 22. Samples of young leaves were collected on the following localities: the town of Srebrenik, the town of Gradačac, the municipality of Čelić-Tuzla Canton, Bosnia and Herzegovina, in the spring 2019.

For each examined variety, 10 healthy, undamaged leaves were taken. The plant material was dried in a digester with a

homogeneous air flow for 10 days, in genetic laboratory, Department of Biology, Faculty of Science, University of Tuzla. The samples were then transported in paper bags with silica beads to the INGEB Institute of Genetic Engineering and Biotechnology, Sarajevo, for further molecular genetic analysis.

B. Isolation of Genomic DNA from Young Pear Leaves

After fragmentation of the plant tissue, genomic DNA isolation was performed with Soltis Lab CTAB DNA kit according to the manufacturer's protocol. In the first isolation step, the samples were incubated in lysis buffer overnight. Then after adding 24:1 chloroform:Isoamyl alcohol the samples were centrifuged. The upper phase of the supernatant was transferred to a new tube, and after the addition of 7.5M ammonium acetate and isopropanol placed on ice and centrifuged again. The precipitate was washed with 70% and absolute ethanol, followed by drying of the DNA precipitate at the bottom of the tube. DNA was dissolved in ddH₂O and used for amplification.

C. SSR Analysis

For DNA amplification, the KAPA3G Plant PCR kit (Kapa Biosystems) was used according to the appropriate manufacturer's protocol. Polymerase chain reaction (PCR) amplification of microsatellite sequences took place in the GeneAmp PCR System 9700 (Applied Biosystems, United States). To confirm the authenticity of varieties and determine the genetic diversity, twelve microsatellite markers used in studies on peach genotyping were used: CH02b10, CH03g07, EMPC117, CH05e03, CH02CO2a, EMPC11, CH03D12, CH02C11, CH01d03, CH04e03, CH01d09 and CH01f07a. The names and sequences of the primers, as well as the conditions for carrying out PCR are described earlier (28,29,30). Detection of DNA profiles and fragment sizes was performed using fluorescently labeled primers with the internal standard GeneScan-500 LIZ, and PCR products were analyzed on an ABI PRISM®3500 automatic sequencer (Genetic Analyzer 3500, Applied Biosystems) with 8 capillaries 50 cm long, which enables separation and analysis of DNA fragments according to the principle of capillary electrophoresis and as detection by CCD camera. All electrophoresis data were collected in the ABI PRISM® Collection software.

D. Biostatistical Analysis

Biostatic analysis involved calculating the number of detected alleles (DA) and expected (EH) heterozygosity [31] in computer program SPAGeDI 1.3, [32]. The effective allele number (AE) was calculated in the computer program GenAlEx [33], UPGMA cluster analysis based on the Jaccard similarity coefficient was used to determine similarities between genotypes and was performed in the computer program MEGA 6 (Molecular Evolutionary Genetics Analysis) [34].

III. RESULTS AND DISCUSSION

In all analyzed microsatellite locus, amplification was successful. In general, the size of the amplified DNA fragments scored ranged from 95 to 254bp, are described earlier [28]-[30]. Detection of DNA profiles and fragment

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Genetics Analysis) [34]. Genetic profiles of nine tested pear samples, based on 12 analyzed microsatellite locus are shown in Table I. Effective number of alleles (EA), number of detected alleles per locus (DA), ratio between effective and detected number of alleles per locus (EA/DA) and expected (EH) heterozygosity for 12 SSR locus are also shown in Table II. Among the investigated samples, the mean values of detected alleles (DA) and effective alleles (EA) were 6.30 and 4.70, respectively. The expected heterozygosity (EH) was within 0.67–0.92.

TABLE I: GENETIC PROFILES OF NINE TESTED PEAR SAMPLES, BASED ON 12 ANALYZED MICROSATELLITE LOCUS

SAMPLE	CH02b10	CH02b10	CH03g07	CH03g07	EMPC117	EMPC117	CH05e03	CH05e03	CH02C02a	CH02C02a	EMPC11	EMPC11	CH03D12	CH03D12	CH02C11	CH02C11	CH01d03	CH01d03	CH04e03	CH04e03	CH01d09	CH01d09	ECH01f07a	ECH01f07a
Okrugla	112	155	200	240	95	119	178	178	128	142	134	134	107	111	222	222	135	150	175	194	127	145	183	197
Karamanka	140	155	230	240	95	115	174	174	130	130	148	148	107	123	213	239	135	150	178	185	154	154	190	190
Ječmenka	140	155	230	242	113	135	162	162	128	134	136	136	107	119	235	235	133	150	175	178	129	143	190	190
Huremka	155	155	242	242	113	115	172	172	128	128	148	148	119	125	217	237	153	153	178	197	131	164	180	190
Savka	155	155	240	240	119	119	188	188	128	128	136	136	107	119	222	235	133	153	173	178	127	145	192	190
Pšeničnjača	110	155	200	240	117	123	188	188	128	128	140	144	107	111	222	235	133	153	173	194	133	145	183	190
Kongreska 22	110	128	224	254	107	111	164	164	130	144	144	144	107	125	213	237	135	153	185	190	137	155	180	183
Žutica	112	155	200	242	113	119	188	188	128	134	144	144	107	111	222	222	133	150	173	173	127	145	183	195
Mesnjača	128	140	224	230	111	111	164	164	128	144	148	148	109	125	215	237	133	153	178	185	133	155	183	190

TABLE II: RANGE OF DETECTED ALLELES, NUMBER OF DETECTED ALLELES (DA), EFFECTIVE NUMBER OF ALLELES (EA), RATIO (RATIO) BETWEEN EFFECTIVE AND DETECTED NUMBER OF ALLELES (EA / DA), EXPECTED (EH) HETEROZYGOSITY FOR 12 SSR LOCUS ON NINE TESTED SAMPLES PEARS

Locus	Range	DA	EA	EA/DA	EH
CH02b10	110/155	5,0	3,2	0,64	0,73
CH03g07	200/254	6,0	5,1	0,85	0,85
EMPC117	95/135	9,0	7,0	0,78	0,91
CH05e03	162/188	6,0	4,8	0,80	0,84
CH02C02a	128/144	5,0	2,7	0,54	0,67
EMPC11	134/148	5,0	4,0	0,80	0,79
CH03D12	107/125	6,0	4,2	0,70	0,80
CH02C11	213/239	7,0	4,8	0,69	0,84
CH01d03	133/153	4,0	3,8	0,95	0,78
CH04e03	173/197	7,0	5,4	0,77	0,86
CH01d09	127/164	10,0	7,7	0,77	0,92
CH01f07a	180/197	6,0	3,4	0,57	0,75
Average		6,3	4,7	0,75	0,81

Fig. 1. UPGMA cluster analysis based on Jaccard's similarity coefficient, performed based on polymorphism of 10 SSR locus within nine apple samples.

The obtained dendrogram indicates a pronounced differentiation, i.e., each of the nine examined varieties represents a unique genotype. Assessment of genetic diversity within a cultivated crop has important consequences in breeding and the conservation of genetic resources.

IV. CONCLUSION

The results of the research enabled the identification of 9 analyzed autochthonous pear varieties from the Tuzla Canton in Bosnia and Herzegovina: Mesnjača, Žutica, Ječmenka, Pšeničnjača, Savka, Okrugla, Karamanka, Huremka and Kongreska 22 as unique genotypes. Among the nine samples examined, there were no cases of synonyms or homonyms. The examined sets of genotypes possess significant genetic variability, which is especially important because relatively small number of samples were analyzed. The autochthonous pear varieties analyzed in this paper represent an interesting genetic resource, with useful agronomic traits that can be used in future cultivation. Such results certainly impose the importance of their adequate preservation.

COMPETING INTERESTS

All the authors declare that they have no conflict of interest.

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