



Short communication

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## Genetic characterization of autochthonous varieties of "Lubeničarka" pear using AFLP markers

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### Abstract

In this paper, we present the results of the examination of the genetic characteristics of 7 autochthonous varieties of "Lubeničarka" pear (watermelon pear) that are typical for the Banjaluka region, using AFLP molecular markers. In order to reliably confirm that there are differences among selected varieties we have analyzed their genetic profiles using AFLP genetic markers and established, based on the Jaccard similarity coefficient, that there is a genetic variability among the studied varieties. Furthermore, based on these analyzes we have classified these varieties into 3 groups of which variety G\_19 has a very large coefficient (0.4369) when compared to other varieties. These results might be immensely important for present and future pear breeding and genetic improvement program.

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### Keywords

*Lubeničarka, genetic characterization, molecular marker, pear*

### Introduction

The pear (*Pyrus communis* L.) is one of the most important fruit trees, having been cultivated in Europe and Asia for at least two thousand years and is presently commercially grown in all temperate regions. Fruit biodiversity in Bosnia and Herzegovina

is reflected in many autochthonous varieties like plums, apples, pears, sweet cherries, as well as peaches, etc. Many autochthonous varieties of our region are of unknown origin. It is believed that they were brought in during some of the numerous migrations of human populations in this region. Many varieties of fruits have adapted and acquired new characteristics, while some have remained unchanged. Compared to modern varieties of fruits,

autochthonous varieties are more diverse and more resistant to biotic and abiotic factors (Ognjanov et al., 2000). Watermelon (Lubeničarka) pear, sometimes called "Bostanjača" pear, is very popular among local people because of its good taste. It is used for fresh consumption and home processing and has good resistance to common diseases and pests as well as high tolerance to low temperatures. "Lubeničarka" pear is recognizable by their characteristic red color of the fruit flash which is similar to ripe watermelons.

Beširević (2009), described around 100 autochthonous varieties of apples and pears from the territory of Bosnia and Herzegovina. He stated that this area is favorable for fruit production, due to a very favorable climate and precipitation regime. Beširević also noted that for a large number of local varieties of apples, pears and other fruits there are no unique names. Pomological characterization of pears from the Lubeničarka group was completed by Mičić et al. (2012) for three varieties of pears from Bosnia and Herzegovina. Variety Krupna Lubeničarka was recommended for further production at the beginning of XX century. The variety was grouped with two others (Crna Lubeničarka and Bijela Lubeničarka) under the common name Lubeničarka. The results of the study showed that the variety Krupna Lubeničarka has vegetative progeny with stable pomological characteristics that are clear and reliable characteristic of this variety (Mičić et al., 2012). Genotypes Crna and Bijela Lubeničarka have certain pomological differences that clearly distinguished them from one another. However a certain similarity between these varieties raised the question about their reliable pomological and genetic characterization (Radoš et al., 2017; Kajkut et al. 2015; Šebek et al., 2014; Mičić et al. 2012).

Genetic characteristics of pear have not been fully identified due to its low morphological diversity, lack of differentiating characters within species and widespread crossability. Therefore, estimation of genetic diversity among *Pyrus sp.* is often very difficult. Classical methods of describing morphological properties include certain deficiencies, such as: objectivity in identification, great similarities among the cultivars, the connection of the analysis for the fruiting period, plant age, etc.

Introduction of PCR technology has routinized molecular identification, characterization and genotyping of many fruit species including pear (Akçay et al., 2014; Liu et al., 2015). Amplified Fragments Length Polymorphism (AFLP) is a DNA fingerprinting technique developed by Zabeau & Vos (1993) and Vos & Kuiper (1997). AFLP markers are genomic restriction fragments detected after selective amplification using the polymerase chain reaction (PCR). The largest number of research with the application of AFLP markers was implemented for pear (Shaymaa et al., 2018; Wolf et al., 2017; Bao et al., 2008; Monte-Corvo et al., 2000; Vos et al., 1995).

The present study has been conducted with the main objectives to determine the genetic difference of between 7 of autochthonous varieties of "Lubeničarka" pear using Amplified Fragments Length Polymorphism markers.

## Material and methods

### *Plant material*

Fresh pear leave samples were collected from seven autochthonous varieties of pear (variety G\_14 'Lubeničarka' town Banja Luka (Hisete), variety G\_17 'Lubeničarka' town Banja Luka (Bistrica), variety G\_15 'Lubeničarka' municipality Srbac, variety G\_16 'Lubeničarka' municipality Srbac, variety G\_18 'Prava Lubeničarka' municipality Prnjavor (Crnadci), variety G\_19 'Obična Lubeničarka' municipality Prnjavor (Orašje) and variety G\_20 'Krupna Lubeničarka' municipality Prnjavor (Kokori). Genetic analysis have been carried out at the Faculty of Agriculture in the Department of Plant Breeding, Genetics and Biometrics, University of Zagreb.

### *DNA isolation*

Genomic DNA was isolated from young leaf tissue previously dried by lyophilisation. After lyophilisation the tissue was ground into a fine powder, and DNA isolation was performed according to the manufacturer's instructions of the DNeasy® Plant Mini Kit for the isolation of DNA from plant tissues (Qiagen, 2015).

### AFLP analysis

Based on the determined concentration of DNA of sample, dilution on the working concentration of 25 ng/μl for further AFLP analysis was done. AFLP analysis, with the following modifications, was carried out according to the protocol described by Vos et al. (1995). Restriction of the DNA was performed in a total volume of 20 μl by using the restriction enzymes EcoRI (New England Biolabs), characterized by a specific recognition site with six bases (the so-called "rare cutter") and MseI (New England Biolabs), characterized by a specific recognition site with four bases (the so-called "frequent cutter") with addition of S.C.-NEB buffer which is specific for the listed enzymes. Digestion was carried out by mixing of 3 μl of the restriction enzymes (5U/μl) with 17 μl of a DNA (concentration of 25ng/μl). The digestion lasted for about an hour at a temperature of 37°C in a thermostat Therma Stat Plus (Eppendorf). The result of restrictions has been tested on standard 0.8% agarose gels, loading 2.5 μl of solution of DNA restriction. The remaining volume of 17.5 μl of a restriction solution was mixed with 7.5 μl of a ligation mixture containing 5 pmol EcoRI adapter and 50 pmol MseI-adapter and with 1 U T4V DNA ligase (New England Biolab), 1.2 mM ATP and buffer composition comprised of 10 mM TRIS-HAc, 10 mM MgAc, 50 mM Kac and 5 mM DTT. The incubation lasted three and a half hours at a temperature of 37° C. Adaptors are composed of two mutually homologous primers in their middle part, while the base ends of the primer are homologous with a specific base sequences (the so-called "sticky ends") at the ends of restriction fragments created by the restriction.

EcoRI adapter consists of two primers with sequences: 5-CTCGTAGACTGCGTACC and CTGACGCATGGTTAA-5, and MseI-adapter primers: 5-GACGATGAGTCCTGAG and TACTCAGGACTCAT-5. Pre-amplification was carried out in a total volume of 20 μl with composition of 1/4 of restriction fragments (i.e. 5 μl of a restriction solution mix), to which was added adapter by ligation in a buffer composed of 20 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub> and 50 mM KCl, with 0.2 mM of each dNTP, 0.5 U Taq-polymerase (Sigma),

and 0.25 μM of each pre-amplification primer (E01 and M02). Selected primers are complementary to EcoRI and MseI adapters, and one additional selective base allows amplification of 1/16 of restriction fragments. Pre-amplification was performed in Veriti™ 96-well Thermal Cycler (Applied Biosystems), according to the regime: [92°C/60s, 60°C/30s, 72°C/60s.] 25x. Products of pre-amplification were diluted in a ratio 1:25 and as such have been used for the selective pre-amplification. Selective pre-amplification was carried out in a total volume of 20 μl (1/4 of the volume, i.e. 5 μl of the pre-diluted product of pre-amplification) of the buffer composition of 20 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub> and 50 mM KCl with 0.2 mM of each dNTP, 0.4 U Taq-polymerase (Sigma), 0.25 μM "E+3" and "M+3" primers. Primers E are marked by specific colours 6Fam, Ned and Vic for laser load on the device for capillary electrophoresis (Genetic Analyser 3130, Applied Biosystems). 'E+3' and 'M+3' primers have the same sequence as the primers used in pre-amplification, and each of them have three selective basis which enables further selective amplification only for 1/256 of all the fragments of pre-amplification. Adapter sequences, primers sequences and their base sequences used in the AFLP analysis are shown in table 1.

Selective amplification was carried out by Veriti™ 96-well thermal cycler (Applied Biosystems), according to the touch down regime:

94°C/30 s. - [94°C/30 s. - 65°C -0.7°C/cycle -  
72°C/60 s.]11x -  
- [94°C/30 s. - 56°C/30 s. - 72°C/60 s.]24x - 72°C/5  
min.

The products of amplification proceeded by capillary electrophoresis *Genetic Analyser 3130* (Applied Biosystems), and visualized using the software package *Gene Mapper® ver. 4.0.* (Applied Biosystems).

### Statistics

For further analysis only indisputable visual fragments appeared as a clear signal (amplitude) of each AFLP fragments were taken into account. The presence of the band is marked with 1, and its absence with 0. The binary matrix of molecular data

Table 1. Overview of the primers used in the ALFP analysis

Primer code		The primers used in the analysis after screening ↓↓↓
<b>Adapter <i>EcoRI</i></b>	5'- ctc gta gac tgc gta cc -3' 5'- aat tgg tac gca gtc -3'	6Fam E32 - M47 (p1) 6Fam E33 - M48 (p2) 6Fam E32 - M49 (p3)
<b>Primer E+1 (pre-amplification)</b>	E01 5'- gac tgc gta cca att c a -3'	6Fam E32 - M59 (p4)
<b>Primer E+3 (selective amplification)</b>	E32 5'- gac tgc gta cca att c aac -3'	6Fam E33 - M60 (p5)
	E33 5'- gac tgc gta cca att c aag -3'	6Fam E32 - M61 (p6)
	E35 5'- gac tgc gta cca att c aca -3'	Ned E35 - M50 (p7)
	E37 5'- gac tgc gta cca att c aca -3'	Ned E35 - M47 (p8)
	E40 5'- gac tgc gta cca att c agc -3'	Ned E37 - M59 (p9)
<b>Adapter <i>MseI</i></b>	5'- gac gat gag tcc tga g -3' 5'- tac tca gga ctc at -3'	Vic E40 - M61 (p10) Vic E40 - M50 (p11) Vic E40 - M60 (p12) Vic E40 - M62 (p13)
<b>Primer M+1 (pre-amplification)</b>	M02 5'- gat gag tcc tga gta a c -3'	Vic E40 - M59 (p14)
<b>Primer M+3 (selective amplification)</b>	M47 5'- gat gag tcc tga gta a caa -3'	
	M59 5'- gat gag tcc tga gta a cta -3'	
	M60 5'- gat gag tcc tga gta a ctc -3'	
	M61 5'- gat gag tcc tga gta a ctg -3'	
	M62 5'- gat gag tcc tga gta a ctt -3'	

was used for further statistical analysis in the order to calculate Jaccard coefficients of similarity (difference) (Jaccard, 1908). Based on obtained coefficients the cluster dendrogram of similarity of the studied genotypes was made.

## Results and Discussion

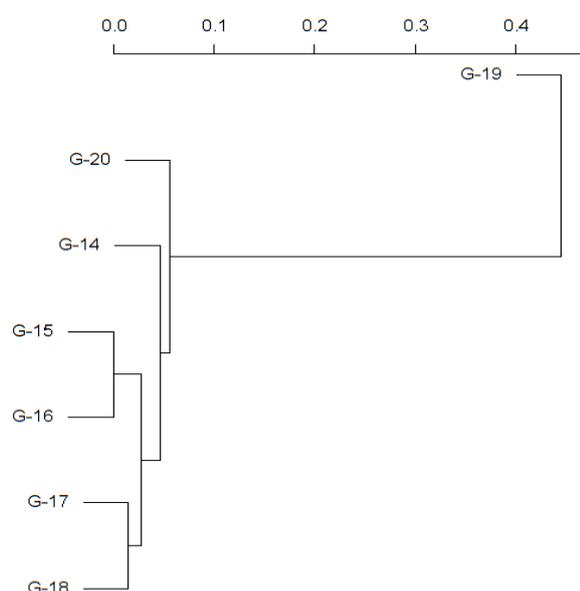
AFLP fingerprinting was carried out for 7 autochthonous varieties of pear samples analyzed in the current study. This molecular technique is considered to be the most effective method in examining genotype of *Pyrus communis* L. (Monte-Corvo et al., 2002). Genetic identification of selected varieties of Lubeničarka pears was done using AFLP markers, and the results are presented in figure 1. We examined 25 combinations of primers, of which 13 polymorphic were selected and were applied to all samples included in the study. Tested

varieties were grouped in 3 groups. The first group with no observed differences in the genetic profiles, was with varieties G\_15 and G\_16 (coefficient of diversity (0.000)). The second group consists of varieties G\_17 and G\_18 with the observed difference of 0,015. Especially interesting was the group with varieties G\_14 and G\_20 and a coefficient of difference of 0,056. Complete separation from this group of varieties showed the variety G\_19 with the observed difference of 0,432 from the nearest tested variety (Table 2). Based on the calculated coefficients of difference, the variations between the observed varieties demonstrated the existence of 3 groups of varieties. The group I consists of only variety G\_19 which indicates that this variety most probably does not belong to the Lubeničarka type. Varieties G\_15, G\_16, G\_17 and G\_18 make the II group, while III group comprises of variety G\_14 and G\_20.

**Table 2.** Coefficient of diversity according to Jaccard

Accessions	G_14	G_15	G_16	G_17	G_18	G_19
G_15	0.039					
G_16	0.039	0.000				
G_17	0.046	0.022	0.022			
G_18	0.037	0.027	0.027	0.015		
G_19	0.437	0.437	0.437	0.446	0.436	
G_20	0.056	0.038	0.038	0.054	0.045	0.432

Comparing our results obtained by genetic analysis and the classification based on morphological analysis observed by Mičić et al. (2012), observed varieties from group II belong to Crna or Bijela Lubeničarka types and varieties from Group III belong to Krupna Lubeničarka type. Regarding these studies the conditions created for the precise identification of different varieties of Lubeničarka pears in the appropriate groups are the prerequisite for using of genofond of Lubeničarka pear for different purposes.



**Figure 1.** The dendrogram grouping the genotypes in accordance with the coefficients of difference

The AFLP technique was confirmed to be an efficient tool for genotyping and estimating genetic variation in pear cultivars. In order to reliably prove that there are differences among selected varieties we have analyzed the genetic profiles using AFLP genetic markers and established, based on the Jaccard similarity coefficient, that there is a genetic variability among the studied varieties. Furthermore,

based on these analyzes we have classified these studied varieties into 3 groups of which variety G\_19 has a very large coefficient (0.4369) when compared to other varieties, so that the affiliation of this variety to Lubeničarka variety stays questionable. Based on the calculated coefficients of difference, the variations between the studied varieties demonstrated existence of 3 groups of varieties. The group I consists of only variety G\_19 indicated that this variety the most probably does not belong to the Lubeničarka type. Varieties G\_15, G\_16, G\_17 and G\_18 make the II group, while III group comprises varieties G\_14 and G\_20. Overall, it can be concluded that there was polymorphism among the studied varieties. Also, it can be stated that the AFLP was a reliable and a good technique in genotyping and discriminating of respective pear varieties.

## Conclusion

Banja Luka region is characterized by a very rich and varied diversity of old and autochthonous varieties of pears, which represent a very important genetic potential for future breeding programs. Despite the fact that morphological characteristics of these autochthonous varieties of "Lubeničarka" pear were not included in this research, since they were performed before this molecular identification. Based on everything mentioned before, it is necessary to state that phenotype appearance of the analysed varieties was supported and confirmed by this genetic characterization. AFLP analyses confirmed the presence of polymorphism between analyzed varieties pear in Banja Luka region. Based on the calculated coefficients of difference, analyzed varieties were grouped in 3 groups. All genetic profiles of the analyzed varieties belong to the Lubeničarka type, only variety G\_19 indicated that

this variety the most probably does not belong to the Lubeničarka type.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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