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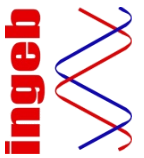

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*Ophrys sphegodes* Mill.*,* in the vicinity of Stolac, Herzegovina. Provided by: *Faruk Bogunić.*

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www.ingeb.unsa.ba
Phone: +387 33 220-926
Fax: +387 33 442-891
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*Review*

# AN ETHNIC GENETICS – CASE STUDY OF B&H: FACTS, FALLACIES AND MYTHS

Rifat Hadžiselimović[1*]

[1]University of Sarajevo, Institute for Genetic Engineering and Biotechnology, Sarajevo, Bosnia and Herzegovina

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***Correspondence**
E-mail: rifat.hadziselimovic@ingeb.unsa.ba

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*Population genetics, Genetic distance, Balkans ethnic groups, Pseudoscience*

## Abstract

The post-war period (1996 to presence) in the Western Balkans is colored by a kind of competition among (pseudo) scientists and self-proclaimed experts in search for the deepest roots of a particular ethnic group. General conclusions have been reached based of the distribution of a single or only few genetic markers, with no reference to the specific pheno-genotype system studied. The conclusions were all biased by earlier misconceptions and myths about the successive colonization of the Balkans and the inter-genetic relationships among regional populations. In this paper we elaborate methodology and limitations and misconceptions that arise from unsubstantiated use thereof.

## Introduction – Population genetics in B&H timeline

Since its onset, the anthropogenetic research in the Balkans has been carried out in several stages with ever increasing levels of complexity and comprehensiveness. The first demographic-genetic surveys in B&H were published in the last decade of the 19th century by two Austrian military physicians with the rank of "hauptmann" Heinrich Himmel (Herzegovina recruits) and "obersturmarzt" Augustin Weissbach (B&H recruits) (Weisbach, 1895). The following stage was featured by the analysis of individual phenotypic systems with

basic analysis of gene and phenotype frequencies. Periods 1934-1935 and 1953-1966 were dedicated to the analysis of ABO, Rh and MN systems (Kalić and Kostić, 1934; Bošković 1965, Berberović 1968). In 1967-1976 populational studies advanced with respect to the number of markers observed and the level of complexity of mathematical models applied. Numerous biochemical-physiological and morphological characteristics were observed in predominantly urban (sub)populations. Complex study of interpopulational genetic distance was introduced in 1977 with a shift of focus from urban to predominantly non-migratory rural populations (Hadžiselimović, 1981, 1983, 1984).

At the end of this research cycle, the possible relationships between the observed indicators of genetic distance and its possible causes were also examined. This is primarily related to the degree of isolation of local populations. The frequency of marital distance „zero“ (marriage couple originated from the same local population), distribution of the mean marital distance, endogamy coefficient, differential reproductive rate, population size, and correlation between mean geographical distance and mean genetic distance. The highest positive (significant) correlation ($p<0,05$) was observed between genetic distance and population size. However, the largest (significant also) negative correlation was observed between the average genetic distance and marital distance „zero“(0). The overall results indicate that the studied populations constitute „inland islands“ in the sense that the married couples originated from the same local community in 90-98% (Hadžiselimović and Zovko, 1987). From 2002 onwards, population studies include direct genetic markers (nuclear and mitochondrial). The largest dataset (N = about 20,000) was collected for the purpose of DNA profiling of the remains of war victims and consisted of STR profiles of exhumed victims and reference profiles of their surviving relatives. Genetic distance among B&H local and metapopulations, with regard to several (nuclear and mitochondrial) genetic markers was extensively studied by numerous authors (Marjanović et al. 2005a, 2005b, Battaglia et al 2009, Pojskic et al 2013a, 2013b, Ahmić et al. 2014, Kapur-Pojskic et al. 2014, Kovacevic et al., 2014, Sarac et al., 2014, Carreras-Torres et al., 2016, Babić et al., 2017, Pilav et al, 2017, Čakar et al. 2017, Ahmic et al, 2018). Since 2016, contemporary research has gradually been expanding into the field of omic technologies. Following positive experiences in genome analyses (genomics) the first results of transcriptome analysis are expected soon.

**Figure 1.** Eye color distribution in B&H populations (blue, dark and grey-brown), the last decade of the 19th century (Weisbach, 1895)

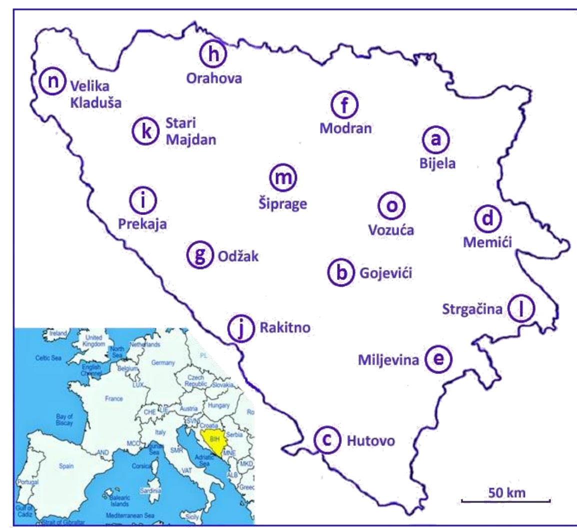


**Figure 2.** Spatial distributiom of 15 local B&H populations studied in the period 1973-1976

## Facts

Previous population-genetic studies of classical morphological, biochemical-physiological traits and molecular-biological markers have not shown any differences in the genetic structure among ethnic populations in Bosnia and Herzegovina. It was

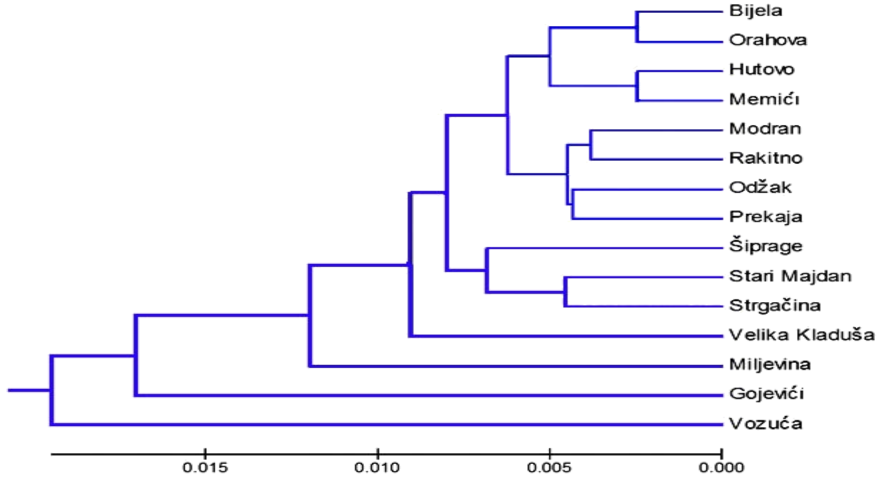


**Figure 3.** Genetic distance among 15 local B&H populations based on 15 phenotypic markers, i.e. the corresponding alleles (ABO blood groups (Io) ABO(H) antigens secretion (se), red (cvp) and green (cvd) color vision, PTC testing (t), tongue rolling (r) and fissuring (f), ear lobe types (l), chin dimple (ch), midphalangeal hairiness (d), crooked 5th finger (rcf), hich-hicker's thumb (dht), proximal thumb joint hyperextensibility (pht), digital index (lf), and long hand nails(ln))

also noted that the differences among local populations within particular ethnic group exceed or are equal to their distance from the said ethnic group as a whole. The latest data, collected through the popular and massive worldwide MyHeritage project (https://www.myheritage.com/), has shed more light on the existing ideas about possible similarities and differences between Balkan populations and ethnic groups. Pojskić et al. (2013) studied the nuclear Alu markers, and reached similar conclusions. Interestingly though, when gender-specific markers (Y-chromosome) were analyzed, the conclusions were still the same (Marjanović et al. 2005a, 2005b). In addition, Pojskić et al. (2013) and other studies (Ahmić, 2005) have established that the net result of genetic diversity study of populations included largely depended on the complex of studied markers. On the other hand, the same authors also concluded that genetic distance estimation heavily depended on the mathematical model used in the evaluation of the observed measures.

## Fallacies – Neglected starting points

"Genetics of Nations"/ Ethnic genetics is a popular syntagma which has entered the scientific vocabulary as well. It is completely unnecessary and has enormous potential for misuse in Western Balkans region. Recent interpretations were based on erroneous premises such as:

- Axiomatic – uncritical acceptance of consensus Ex-Yugoslavian historiography;
- Complete extinction of ancient population and all earlier epochs;
- Complete replacement in the succession of landmark immigration in B&H;
- Population discontinuity of present-day B&H territory;
- Identification of linguistic and other cultural changes with the biological essence of B&H population.

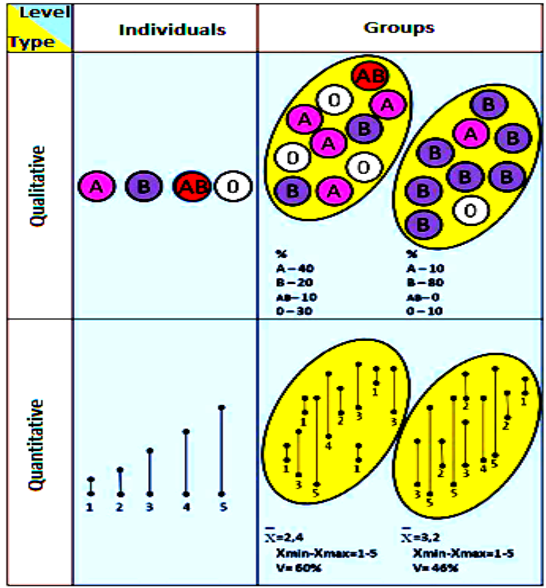


**Figure 4.** Basic parameters of the individual and population differences regarding the qualitative and quantitative variation (Hadžiselimović, 2019)

Striking conclusions are extracted from genetic distance based on a single or few genetic markers.

**Human individual diversity** rests on variation of extremely large number of qualitative and quantitative traits with virtually limitless possibilities to combine their variants (phenotypes).

Even in small group of human individuals, it would be difficult to find two individuals with the same variant of only one trait; even fewer that are matched according to two traits, while those that possess the same combination of any three traits are extremely rare.The number of combinations of variants of dimorphic traits increases according to the geometric progression with 2 as a base (= number of alternative variants) and n as an exponent (= number of traits included). Thus, when combining two traits the number of combinations is 4 (= $2^2$), three traits give 8 combinations (= $2^3$) while four traits result in 16 (= $2^4$) different individual combinations. At the point when we combine 34 traits the number of combination reaches $2^{34}$or over 17 billion, more than 2.5 times the total number of modern inhabitants of the planet, i.e. 1/5 of all people who have seen the light of day on Earth so far. One hundred such properties give the number of combinations far greater than not only the total number of people born so far, but all living beings together. If we factor in the plasticity of genotypes in different environmental scenarios and ontogenetic variability, the number of possible phenotypic combinations in the human population exceeds the total count of electrons in the Cosmos.

**Group or population diversity,** although derived from the individual biological differences, represents a new quality with higher level of organization and complexity. It has specific manifestations and measures; regardless of whether we consider qualitative or quantitative individual variation, group - specific properties inevitably have quantitative (quantitative) parameters. Individuals differ according to the variant or category of particular qualitative or quantitative trait while intergroup differences are commonly defined by the quantity of individual phenotypes of the observed trait. If we take an example of ABO system of blood groups two individuals may differ in the alternative quality (biochemical structure) of ABO antigens so that one individual is group A while the other is group B. However, two hypothetical populations differ in relative frequency of particular blood group so one group contains 40% individuals with blood group A while the other contains 60% of the same variant.

This coarse indicator does not provide even the most elementary information on the intra-group variation of the individuals involved, so the same amounts can occur in extreme cases (absolute homogeneity or heterogeneity). Occurrence of two biologically identical individuals in all of humanity throughout history is infinitely unlikely, thus occurrence of two biologically identical human groups is theoretically impossible.

The genetic characteristics of a human population may rest on:

- the presence of one or more allelogens that are not present in other populations;
- the complete absence of alleles that occur regularly in other populations;
- both of these phenomena;
- general presence but significantly different frequency of individual allelogens in all populations compared.

Neither of the group properties applies to its individual members.

## Myths

Certain ideas about the sequence of settling the area have been propagated for centuries and are consequently deeply rooted in the Balkans.

With this respect, official theories on mass migrations and resettlement have been developed, which have also influenced the official social and political atmosphere. The most prominent tales that reached the level of a myth are:

- Complete Bogomilisation of the parent population (Constituents of AllBosnian Church were erroneously recognized as Bogomils because of dualistic concept of belief);
- Predominant Slavisation of B&H population;
- Existence of a defining genetic marker(s) of a "national/ethnic group" that clearly differentiates a given group from related ethnicities, neighbors and the rest of humanity.

The Balkans is a fertile breeding ground for myths. However, the best historically ingrained myth is the

Serbian one about the „heavenly people". Immediately following the Kosovo battle popular epic poetry emerged that glorified the heroes and vilified the traitors. In the former Yugoslavia (the first of 1918 and the second, socialist one of 1945), this myth was also imposed in all federal units. That syntagma is comparable to the famous Nazi racist thesis on superhumans (*Übermensch*).

Some authors argue that this myth has been developing spontaneously with no interference, until the genocide in Bosnia and Herzegovina. There is also an indication that the Serbian leadership used the myth for political purposes to channel military and paramilitary formations in the direction of ethnic cleansing and war crimes (Anzulović, 1999).

During the War against Bosnia (the most frequently used euphemism is „Bosnian War"), the myth was encouraged by certain Bosnian Serb leaders to describe Bosniaks as "genetic junk" (Statement by Biljana Plavšić given to Belgrade's academic community in 1992.Only Nikola Tucić, academician responded, qualifying the statement as a classic form of racism).

## Facts vs Fallacies and Myths

Analyzing biochemical-physiological characters (BFC), static-morphological characters (SMC) and dynamic-morphological characters (DMC), different clusters were created (Figures 5-10).

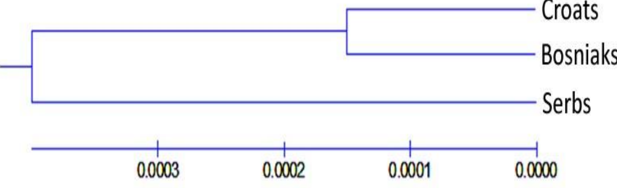


**Figure 5.** UPGMA dendrogram based on the genetic distance analysis according to Reynolds et al. (1983) Subpopulations in B&H: BFC

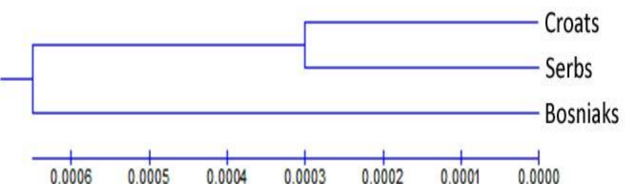


**Figure 6.** UPGMA dendrogram based on the genetic distance analysis according to Reynolds et al. (1983) Subpopulations in B&H: SMC

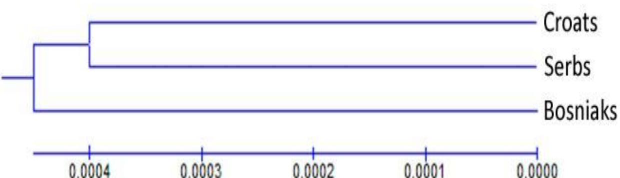


**Figure 7.** UPGMA dendrogram based on the genetic distance analysis according to Reynolds et al. (1983) Subpopulations in B&H: Overall characters observed

The three dendrograms observed exhibit markedly different clustering according to the complexes of the studied traits(figures 5-7). The largest difference with other complexes is shown the in the observed group of the static-morphological properties.

Differences in clustering according to different trait complexes were also observed by Ahmić (2005). She found that the complex of dynamic-morphological traits deviates the most from the other two traitcomplexes: biochemical-physiological and static-morphological (figures 8-10).

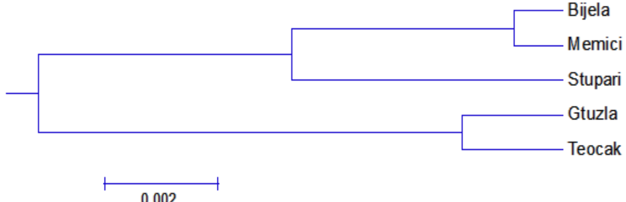


**Figure 8:** Genetic distance relationships with respect to the complex of BFC among the compared populations in Tuzla Region (Ahmić, 2005)

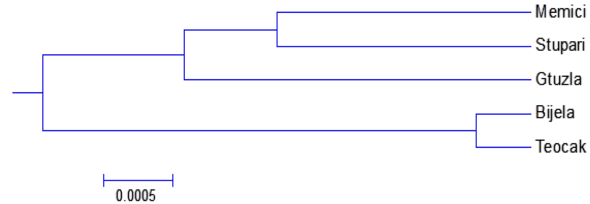


**Figure 9.** Genetic distance relationships with respect to the complex of SMC among the compared populations in Tuzla Region (Ahmić, 2005)

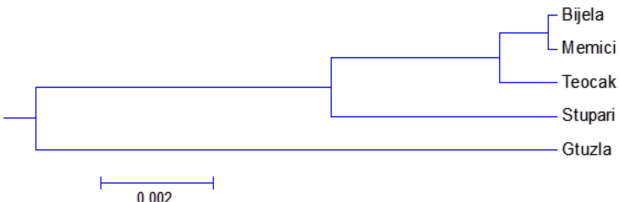


**Figure 10.** Genetic distance relationships with respect to the complex of DMC among the compared populations in Tuzla Region (Ahmić, 2005)

The latest findings on the MyHeritage Platform (with millions of family DNA profiles) have revealed new genetic evidence that does not call into question the indigenous origins of Albanians, Bosniaks, Montenegrins, Serbs, Croats and Macedonians. It has been proven time and again that popular legends about the oldest nations in the Balkans are pure misconceptions, lies and / or superstitions. According to direct genetic markers, the closest ties share Bosniaks, Croats and Serbs, and close to them are Hungarians, Romanians, Bulgarians, Czechs, Slovaks, Poles, Austrians, Greeks, Italians, Germans while Russians, Moldovans and some others populations are only distantly related.

The decades-old myth of the great migration of the Slavs into the Balkan wasteland is overthrown because the so-called “Slavic gene” is slightly expressed in all present-day nations, with relative frequency in:

- Serbs: 30%,
- Croats: 20% and
- Bosniaks: 15%.

According to research conducted by Al Jazeera (2017) Bosnians and Herzegovinians are believed to inherit the remnants of the gene pool of:

- Illyrians: 40%,
- Germans: 20%,
- Celts: 15%, a
- Slavs: 10% .

The population of B&H was not originally constituted in the Slavic but in the Illyrian era, with genetically related haplogroups very similar to Croats and Bosniaks. According to these haplogroups, Turks are twice as close to Albanians as to Serbs and Macedonians, with Bosniaks being the most further of the four populations. No nation in the Balkans has a unique, distinctive haplogroup. That is true for any other European population.

Figure 11, however, clearly shows indicative subclustering (Bosnia and Herzegovina, Slovenia, Austria, Hungary, and Croatia), which particularly singles out a group of countries of fairly heterogeneous geographical, as well as ethnic origin (Pilav et al., 2017).

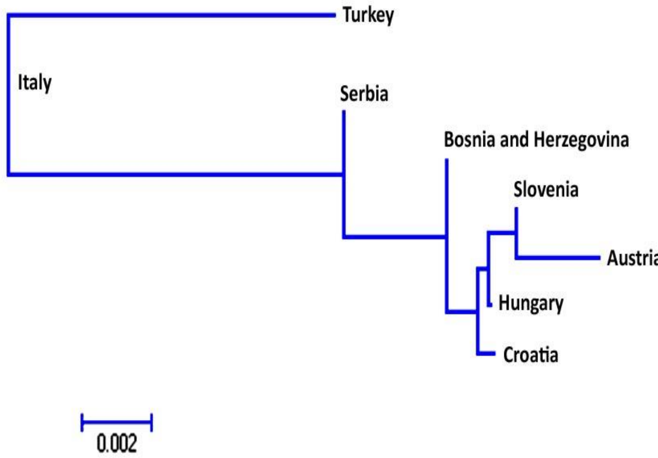


**Figure 11.** UPGMAgenetic distance among selected European population, based on STR markers

## Conclusions

The present-day population of BiH inherits an indigenous gene pool with more or less pronounced "genetic memories" of external invasions, from prehistory and ancient times to the recent epoch. This is not to contradict the fact that it has – in whole or in part – been affixed to various socio-political or appropriate professionally adapted labels. The estimated genetic distance depends primarily on the number and nature of the genetic markers selected, as well as patterns used. Even when the analysis of relationship among the Balkans peoples involved impressively large samples and genetic markers complexes, the general conclusions were drawn overconfidently. Similar attempts were made based on single or few markers with no comment that the presented conclusions exclusively pertain to the studied genetic indicators in the studied samples. Identification of particular gene or genetic marker that would be highly specific for particular nation is both theoretically and practically impossible. Furthermore, there are few markers that cannot be found in most populations.

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*Research article*

# ASSOCIATION BETWEEN KIDNEY INJURY MOLECULE-1 GENE POLYMORPHISM AND ACUTE KIDNEY INJURY AMONG THE LEBANESE PATIENTS

Hajar Karaali[1], Jamilah Borjac[1]*

[1]*Department of Biological Sciences, Faculty of Science, Beirut Arab University, Debbieh, Lebanon*

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## Abstract

Acute kidney injury is a common condition associated with longer hospital stay and increased mortality. Kidney injury molecule-1 (*KIM-1*) is one of the early and sensitive biomarkers for acute kidney injury diagnosis. Therefore we examined the relationship between kidney injury molecule-1 gene polymorphism and acute kidney injury in Lebanese hospitalized patients. Genomic DNA was isolated from blood samples collected from 50 patients and 40 controls. Kidney injury molecule-1 exon 4 was amplified by polymerase chain reaction and the amplified products were sequenced at Macrogen. Serum creatinine and urea levels were measured and compared between controls and patients. Three out of the five known single nucleotide polymorphisms showed significant association with susceptibility to the disease ($P \leq 0.05$). Data analysis implied that carriers of the risk allele of these 3 single nucleotide polymorphisms were more predisposed to acute kidney injury. No association was found between the studied nucleotides variations and creatinine/urea levels. Haplotype analysis showed high association of the block CTA with acute kidney injury incidence and high creatinine and urea levels. Our results suggest that polymorphisms in exon 4 of kidney injury molecule-1 in the Lebanese population may be associated with acute kidney injury.

***Correspondence**

E-mail: j.borjac@bau.edu.lb

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**Keywords**

*Acute kidney injury, Haplotype, Kidney Injury Molecule1, Polymorphism*

## Introduction

Acute kidney injury (AKI) is one of the serious kidney conditions that is associated with increased mortality rate (Uchino et al., 2005). The variability in the prediction of adverse kidney-related outcomes still has no significant explanation. In recent years, the role of genetic single nucleotide polymorphisms as possible determinants of adverse outcomes in AKI patients and the severity of the disease has been the interest of many studies (He et al., 2018; Leung et al., 2009; Susantitaphong et al., 2013).

Due to known limitations in creatinine and urine output tests, the "gold standard" of AKI biomarkers,

new specific, early, and more sensitive biomarkers are needed. Recently, kidney injury molecule-1 (KIM-1) is being considered as a promising biomarker for AKI and kidney failure (Martensson, Martling, & Bell, 2012). *KIM-1*, also known as T cell immunoglobulin and mucin-1 (TIM-1) or hepatitis A virus cellular receptor 1 (HAVCR1), is a type I transmembrane glycoprotein expressed by theproximal tubular epithelial cells (Vaidya, Ferguson, & Bonventre, 2008). The precise role of KIM-1 in renal injury and repair and whether its expression enhances renal protection from nephrotoxic insult is not well established. KIM-1 was shown to play an important role in signaling phagocytosis of apoptotic cells by renal proximal tubular epithelial cells, thus promoting tissue remodeling, restoring tissue normal integrity, and facilitating repair (Rees & Kain, 2008). This molecule was also demonstrated as an early sensitive and specific diagnostic marker for kidney injury in humans and rats exposed to toxic substances (Vaidya et al., 2006).

Genetic polymorphisms of *KIM-1* have major effects on its function in the immune system and thus susceptibility to immune diseases (Meyers et al., 2005). It is important to clarify whether these polymorphisms affect the susceptibility to AKI or to progressive renal injury, and thus may lead to novel therapeutic strategies. Therefore, the aim of this study is to examine the relationship between *KIM-1* gene polymorphism and acute kidney injury in Lebanese hospitalized patients.

## Material and methods

### *Study Population and Data collection*

A total of fifty AKI patients (28 male and 22 females), age range between 18 and 90 years (mean 56.86±20.4 years) were recruited from several hospitals in North Lebanon and from Beqaa between November 2017 and May 2018. Forty non-AKI controls with healthy kidneys (21 male and 19 females) ranging between 19 and 88 years old with mean age of 53±20.3 were recruited from the same regions.

Detailed clinical history records were collected. Kidney-related parameters including serum creatinine and blood urea nitrogen (BUN) were retrieved from hospitals' records.

Patients with AKI were recruited with the help of nephrologists from the hospitals: Tripoli Governmental Hospital (Nephrology and Dialysis department), Orange Nassau Governmental Hospital (Nephrology and Dialysis department), and Bekaa Hospital (Nephrology and Dialysis department). Patients were selected according to the Kidney Disease criteria: Improving Global Outcomes (KDIGO) criteria. AKI patients were defined as patients with at least 1.5-1.9 times or ≥ 0.3 mg/dl increase in serum creatinine from baseline within 48 hours (Kellum, Lameire, & Group, 2013).

Exclusion criteria included participants younger than 18 years, patients undergoing chronic dialysis therapy, pregnant women, and non-Lebanese participants.

The study was approved by the "Institutional Review Board" at Beirut Arab University. Informed consent was obtained from all individual participants included in the study, and a well-designed questionnaire that included baseline demographic characteristics, as well as comorbidity and coexisting conditions was filled.

### *DNA extraction and PCR amplification*

Blood was collected in EDTA tubes from patients and controls during medical visit. Genomic DNA was extracted using the GenElute extracted kit (Sigma–Aldrich, country of origin) according to manufacturer's recommendation. DNA integrity was confirmed by agarose gel electrophoresis while concentrations and purity were determined by spectrophotometric measurement (Evolution 60S UV-Visible Spectrophotometer, Thermo Scientific).

The sequence of primers used to amplify exon 4 of *KIM-1* gene were obtained from Page et al. (Page, Jones, & Stewart, 2006). The sequence of forward primer used was F: 5'-GTTTGACTTATGCTCACTCTC-3' and the reverse primer R: 5'-CCTCACTCTAGACTGTCCTTC-3'.

PCR reaction was performed in a total volume of 25 µl containing 10 ng template DNA , 0.5 µl of forward and reverse primers (10 µM), 12.5 µl of 2x PCR Master Mixture (Sigma-Aldrich) and 10 µl of nuclease- free water. PCR was performed in thermocycler (Thermo Electron Corporation Px2

Thermal Cycler, Biocompare) as follows: 1 cycle at 95˚C for 3 min, followed by 35 cycles consisting of 30 s at 95˚C, 20 s at 50˚C and 30 s at 72˚C and 1 cycle at 72˚C for 4 min. The size of the PCR products obtained was confirmed on 2% agarose electrophoresis and visualized using ChemiDoc-It®2 515 Imager P/N 95-0441-04 UV transilluminator (BIO-RAD, USA). The amplified fragments were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich, USA) and sent for sequencing at Macrogen, South Korea.

*SNP selection*

Single nucleotide polymorphisms (SNPs) were chosen for genotyping based on the NCBI SNP database (http://www.ncbi.nlm.nih.gov). Five *KIM-1* gene SNPs (rs1401877734, rs781388022, rs1259856748, rs1553316, rs12522248) were selected within exon 4 for genotyping.

*Statistical analysis*

All statistical analyses were performed using SPSS version 23 program. Chi-square and One-way ANOVA were used to assess SNPs. Odd's ratio (OR) and 95% interval confidence (CI) were applied to assess risk factors. P-value of ≤ 0.05 was considered significant.

## Results and Discussion

The characteristics of patients and controls are shown in the Table 1. The mean age of participants from both groups of patients and controls were 56.9±20.4 and 53±20.3 years with an average creatinine levels of 3.87±2.15 and 1.01±0.16 mg/dL and with blood urea nitrogen of 140.5 and 23.5 mg/dL, respectively.

**Table 1.** Characteristics of patients and controls

| | AKI patients (*n*= 50) | Controls (*n*= 40) |
|---|---|---|
| Age[a] | 56.9±20.4 | 53.0±20.3 |
| Gender (male/female) | 28/22 | 21/19 |
| BMI[a] | 26.07±4.89 | 27±5.6 |
| Creatinine[a] (mg/dL) | 3.87±2.15 | 1.01±0.16 |
| Blood urea nitrogen[a] (mg/dL) | 140.5±71.17 | 23.5±2.4 |

[a]Values are shown as mean ±standard deviation

We identified 5 SNPs of *KIM-1* that are associated with susceptibility to acute kidney injury. The genotype frequency and allele frequency of these SNPs selected in exon 4 of *KIM-1* gene in AKI patients and control subjects are shown in Table 2.
As shown, 3 SNPs (rs1401877734, rs781388022, rs1259856748) showed significant differences in the genotypic and allelic frequencies between patients and controls (P ≤ 0.05). In this study we report the first evidence that genetic variation in *KIM-1* is associated with susceptibility to AKI in Lebanese population. *KIM-1* gene is located on the chromosomic region 5q33.2. It is a member of a family that plays a critical role in regulating immune response and is linked to susceptibility to autoimmune diseases (Garcia-Lozano et al., 2010). Among its 9 exons, exon 4 is known to be highly polymorphic (Lee et al., 2011). *KIM-1* plays an important role in mediating phagocytosis of apoptotic cells within proximal tubule and facilitating recovery from kidney injury by down regulating the immune response (Yang et al., 2015). The first study conducted in 2003 provided evidence for an association between *KIM-1* polymorphism and human atopic disease (McIntire et al., 2003). Gao et al. showed association between *KIM-1* genetic variation and asthma in African American population (Gao et al., 2005). Similarly, Chae et al. showed the association of 10 novel SNPs in the both promoter and coding region of exon 4 of *KIM-1* gene with allergic diseases (asthma and atopic dermatitis) and rheumatoid arthritis in Korean population (Chae et al., 2003). Previous studies identified associations between AKI and other genes. Isbir et al. found the association between angiotensin-converting enzyme (ACE) and apolipoprotein E (APO E) genes polymorphisms and AKI occurrence after cardiac surgery. However, no association was demonstrated between the disease and angiotensin II type 1 receptor (AGTR1) polymorphism (Isbir et al., 2007). Also, no association was found between AKI incidence and catalase, the oxidative stress gene (Perianayagam et al., 2007).
Similarly, no significant association was obtained between genes involved in inflammatory and anti-inflammatory pathways and AKI (Stafford-Smith et al., 2005; Treszl et al., 2002).

On the other hand, the association between SNPs in the promoter region of TNFα and IL10 and the decreased death risk in AKI patients was demonstrated (Jaber et al., 2004). Likewise, an association between NFKB Inhibitor alpha gene and AKI incidencewas reported (Bhatraju et al., 2015). In the prior studies, none of the mentioned variations were examined in relation to any disease including AKI, except for rs12522248 and rs1553316. Recently, rs12522248 was found to be

**Table 2.** Distribution of the genotype and allele frequencies of *KIM-1 gene* in AKI patients and controls

| | **AKI** *N* (%) | **Control** *N* (%) | **Genotype** | ***P*-values** | **OR (95% CI)** |
|---|---|---|---|---|---|
| rs1401877734: G>C | | | | | |
| Genotype frequency | | | | | |
| GG | 2 (4%) | 22 (55%) | | | Reference Genotype |
| GC | 39 (78%) | 10 (25%) | (GC+CC) vs. GG | **0.000*** | 2.133 (1.507-3.019) |
| CC | 9 (18%) | 8 (20%) | | | |
| Allele frequency | | | | | |
| Allele G | 2 (4%) | 28 (70%) | | | Reference Allele |
| Allele C | 48 (96%) | 12 (30%) | C vs. G | **0.000*** | 3.200 (1.987-5.155) |
| rs781388022: C>T | | | | | |
| Genotype frequency | | | | | |
| CC | 13 (26%) | 30 (75%) | | | Reference Genotype |
| CT | 27 (54%) | 2 (5%) | (CT+TT) vs. CC | **0.000*** | 2.960 (1.689-5.189) |
| TT | 10 (20%) | 8 (20%) | | | |
| Allele frequency | | | | | |
| Allele C | 22 (44%) | 32 (80%) | | | Reference Allele |
| Allele T | 28 (56%) | 8 (20%) | T vs. C | **0.001*** | 2.800 (1.437-5.454) |
| rs1259856748: C>T | | | | | |
| Genotype frequency | | | | | |
| CC | 2 (4%) | 28 (70%) | | | Reference Genotype |
| CT | 33 (66%) | 2 (5%) | (CT+TT) vs. CC | **0.000*** | 3.200 (1.987-5.155) |
| TT | 15 (30%) | 10 (25%) | | | |
| Allele frequency | | | | | |
| Allele C | 3 (6%) | 30 (75%) | | | Reference Allele |
| Allele T | 47 (94%) | 10 (25%) | T vs. C | **0.000*** | 3.760 (2.188-6.461) |
| rs1553316: T>C | | | | | |
| Genotype frequency | | | | | |
| TT | 6 (12%) | 10 (25%) | | | Reference Genotype |
| TC | 1 (2%) | 0 (0%) | (TC+CC) vs. TT | 0.109 | 1.173 (0.955-1.442) |
| CC | 43 (86%) | 30 (75%) | | | |
| Allele frequency | | | | | |
| Allele T | 7 (14%) | 10 (25%) | | | Reference Allele |
| Allele C | 43 (86%) | 30 (75%) | C vs. T | 0.185 | 1.147 (0.929-1.416) |
| rs12522248: A>G | | | | | |
| Genotype frequency | | | | | |
| AA | 41 (82%) | 32 (80%) | | | Reference Genotype |
| AG | 8 (16%) | 4 (10%) | (AG+GG) vs. AA | 0.810 | 0.900 (0.382-2.120) |
| GG | 1 (2%) | 4 (10%) | | | |
| Allele frequency | | | | | |
| Allele A | 49 (98%) | 36 (90%) | | | Reference Allele |
| Allele G | 1 (2%) | 4 (10%) | G vs. A | 0.100 | 0.200 (0.023-1.720) |

*Significant (P ≤ 0.05) values are in bold.

associated with coronary heart disease and ischemic stroke (Zhang et al., 2018). However, this SNP was not associated with systemic lupus erythematosus in Chinese population (Li et al., 2011). In addition, Grabmer *et al*. examined the association of this SNP and rs1553316 with multiple sclerosis in Western Austria, but no significant association was demonstrated (Grabmer et al., 2010).

**Table 3.** Haplotype frequencies in AKI patients and control subjects

| Haplotype | AKI *N* (%) | Control *N* (%) | P-value | OR (95% CI) |
|---|---|---|---|---|
| CTA | 46 (92%) | 4 (10%) | **0.000*** | 0.089 (0.035-0.229) |
| GCA | 2 (4%) | 6 (15%) | 0.132 | 1.129 (0.980-1.302) |
| CTG | 1 (2%) | 2 (5%) | 0.583 | 1.032 (0.951-1.119) |
| CCA | 1 (2%) | 0 (0%) | 1.000 | 0.980 (0.942-1.020) |

*Significant (P ≤ 0.05) values are in bold

In order to investigate the potential association between the studied SNPs and AKI, haplotype analyses were performed. Subsequent haplotype analysis revealed that 3 SNPs (rs1401877734, rs1259856748, rs12522248) formed one haplotype block. An overall of 4 haplotypes were generated, among which only one haplotype (CTA) showed a significant difference between patients and controls and it seems to provide a higher risk for the acute kidney injury occurrence, while the others were not shown to confer a protective effect against the disease. The results are shown in Table 3.We also examined the association between *KIM-1* SNPs and different parameters including age, gender, BMI, blood pressure, smoking, and other diseases, such as diabetes, anemia, liver and pulmonary disease. Differences in these parameters between AKI patients and controls are shown in Table 4. No association was observed (P > 0.05) implying that the effect of *KIM-1* gene polymorphism is independent of these parameters in AKI.

In addition, the association between *KIM-1* genetic polymorphisms and serum creatinine and urea levels was analyzed as shown in Table 5. None of these SNPs was found to be significantly associated with increased creatinine and urea levels in serum. Knowing that creatinine is secreted by proximal tubule (Musso et al., 2009) and that urea is partially reabsorbed by this tubule (Gowda et al., 2010), our results suggest that the genetic polymorphism of KIM-1 may lead to alterations in its genetic expression in the proximal tubules, which in turn may affect creatinine tubular secretion and urea tubular reabsorption and thereby altering their serum levels .Haplotype frequencies among AKI patients and their association with serum creatinine and urea were investigated and shown in the Table 6. Analyzing these haplotypes, only haplotype CTA showed a significant difference between AKI patients and controls (P < 0.05), providing a higher risk for AKI. Although none of the studied SNPs showed a significant correlation with serum creatinine and urea levels, the carriers of CTA haplotype block consisting of three SNPs had the highest levels of these parameters.

**Table 6.** Association between haplotypes in *KIM-1* and serum parameters

| Haplotype | Samples number (%) | Creatinine[a] (mg/dL) | *P*-values | Urea[a] (mg/dL) | P-values |
|---|---|---|---|---|---|
| CTA | 46 (92%) | 2.6±1.14 | **0.009*** | 88.5±18.8 | **0.007*** |
| GCA | 2 (4%) | 1.8±0.9 | 0.999 | 84.0±62.3 | 0.840 |
| CTG | 1 (2%) | 3.5 | 0.755 | 207 | 0.283 |
| CCA | 1 (2%) | 3.08 | 0.917 | 96 | 0.652 |

*Significant (P ≤ 0.05) values are in bold. [a]Values are shown as mean ±standard deviation

**Table 4.** Baseline parameters and clinical data of AKI patients and controls

| Parameters | AKI Patients (*n*=50) | | Control (*n*=40) | |
|---|---|---|---|---|
| | Yes (%) | No (%) | No (%) | Yes (%) |
| Any family member with kidney diseases | 15 (30%) | 35 (70%) | 6 (15%) | 34 (85%) |
| Heart disease | 13 (26%) | 37 (74%) | 16 (40%) | 24 (60%) |
| Diabetes | 12 (24%) | 38 (76%) | 10 (25%) | 30 (75%) |
| Blood pressure problems | 20 (40%) | 30 (60%) | 20 (50%) | 20 (50%) |
| Anemia | 17 (34%) | 33 (66%) | 14 (35%) | 26 (65%) |
| Severe dehydration | 9 (18%) | 41 (82%) | 8 (20%) | 32 (80%) |
| Weak immunity | 10 (20%) | 40 (80%) | 6 (15%) | 34 (85%) |
| Liver problems | 2 (4%) | 48 (96%) | 6 (15%) | 34 (85%) |
| Pulmonary disease | 9 (18%) | 41 (82%) | 12 (30%) | 28 (70%) |
| Vision problem | 19 (38%) | 31 (62%) | 22 (55%) | 18 (45%) |
| Any surgery or organ transplantation | 30 (60%) | 20 (40%) | 22 (55%) | 18 (45%) |
| Received blood transfusion | 23 (46%) | 27 (54%) | 14 (35%) | 26 (65%) |
| Currently a smoker | 19 (38%) | 31 (62%) | 20 (50%) | 20 (50%) |
| Drink caffeinated beverages | 40 (80%) | 10 (20%) | 32 (80%) | 8 (20%) |
| Drink alcohol | 0 (0%) | 50 (100%) | 2 (5%) | 38 (95%) |

**Table 5.** Differences in the value of serum creatinine and urea among AKI patients stratified by different *KIM-1* genotypes

| SNP | Genotype | Samples number (%) | Creatinine[a] (mg/dL) | P-values | Urea[a] (mg/dL) | P-values |
|---|---|---|---|---|---|---|
| rs1401877734: G>C | GG | 2 (4%) | 3.15±0.66 | 0.71 | 115.5±31.45 | 0.569 |
| | GC | 39 (78%) | 3.85±0.33 | | 133.3±10.58 | |
| | CC | 9 (18%) | 4.1±0.82 | | 177.22±28.15 | |
| rs781388022: C>T | CC | 13 (26%) | 2.96±0.15 | 0.228 | 107.01±12.2 | 0.082 |
| | CT | 27 (54%) | 4.29±0.46 | | 146.96±12.99 | |
| | TT | 10 (20%) | 3.91±0.76 | | 166.5±26.84 | |
| rs1259856748: C>T | CC | 2 (4%) | 3.15±0.68 | 0.71 | 115.5±32.44 | 0.569 |
| | CT | 33 (66%) | 4.01±0.38 | | 142.67±11.82 | |
| | TT | 15 (30%) | 3.66±0.5 | | 139.07±20.52 | |
| rs1553316: T>C | TT | 6 (12%) | 3.1±0.27 | 0.612 | 105.17±14.65 | 0.250 |
| | TC | 1 (2%) | 2.4 | | 71 | |
| | CC | 43 (86%) | 4.01±0.33 | | 147.05±11.05 | |
| rs12522248: A>G | AA | 41 (82%) | 3.8±0.32 | 0.495 | 130.1±10.34 | 0.052 |
| | AG | 8 (16%) | 4.26±0.9 | | 185.5±31.14 | |
| | GG | 1 (2%) | 3.5 | | 207 | |

[a]Values are shown as mean ±standard deviation

## Conclusion

This is the first study which showed the association between polymorphisms in *KIM-1* and AKI. In this study, differences in the genotypic and allelic frequencies of *KIM-1* SNPs between controls and patients were obtained. Three SNPs and the CTA haplotype within exon 4 of *KIM-1* are shown to be associated with susceptibility to AKI in Lebanese population. The mechanism by which KIM-1 polymorphism contributes to conferring susceptibility to acute kidney injury remains to be elucidated. However, the present study suggests that KIM-1 may be useful prognostic factor for acute kidney injury. Future studies, with a larger number of participants are needed to clarify the role of genetic variation in determining the risk of AKI among the Lebanese and other populations.

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## Conflict of Interest

The authors state there is no conflict of interest.

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Research article

# RETROSPECTIVE EVALUATION OF CHROMOSOME 1 ANOMALIES IN HEMATOLOGIC MALIGNANCIES: A SINGLE CENTER STUDY

Süreyya Bozkurt[1*], Müfide Okay[2], Filiz Sağlam[3], İbrahim C Haznedaroğlu[2]

[1]Department of Medical Biology, Faculty of Medicine, Istinye University, Istanbul,Turkey
[2]Department of Internal Medicine, Division of Hematology, Hacettepe University Ankara, Turkey
[3]Department of Microbiology, Faculty of Medicine, Istinye University, Istanbul,Turkey

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***Correspondence**
E-mail: sbozkurt@istinye.edu.tr

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## Keywords

*Chromosome 1, hematologic malignancies, translocations*

## Abstract

Various anomalies of chromosome 1 which is the largest chromosome of the human karyotype were found in various hematologic diseases. In this retrospective study, clinical features and cytogenetic anomalies of 35 hematological patients with various chromosome 1 anomalies were correlated. Also the effect of chromosome 1 anomalies to the disease prognosis of those patients was discussed. Conventional cytogenetic analysis of those patients was performed by investigating metaphases of 24 hours stimulated bone marrow samples. After cell culturing, the samples were treated with trypsin and stained with Giemsa (GTG Banding). Analyses were performed on image analysis system. Chromosome 1 anomalies were determined in 35 patients (0.5 %) among 6865 samples having done their conventional bone marrow cytogenetic analysis in our center between January 2008 and March 2016. The ratio of chromosome 1 anomalies of totally 701 anomalies among 6865 patients was 4.9%. Chromosome 1 anomalies were found mostly in patients with Multiple Myeloma (MM), Myelodysplastic syndrome (MDS) and Acute Myeloid Leukemia (AML) in our study group. The most common anomaly was deletion 1 which was seen in 16 (37%) patients. Second most common anomaly was derivation 1 which was seen in 13 (30%) patients. Also translocations between chromosome 1 and other chromosomes were observed. The genetic aberration formed as a result of chromosomal anomalies result in the formation of hematologic malignancies. The effect on disease pathogenesis and prognosis of some of those anomalies are unknown and have to be investigated and determined.

## Introduction

Conventional cytogenetics is useful both diagnostically, such as for the diagnosis of chronic myelogenous leukemia (CML) and for the determination of subgroups of myelodysplastic syndromes (MDS) and acute myeloid leukemia

(AML). Besides, it is important for the determination of disease prognosis and response to treatment (Wan, 2014; Komanduri et al.,2016).
Chromosome 1 is the largest chromosome among all human chromosomes. Various anomalies of chromosome 1 were found in various hematologic diseases. These can be structural anomalies such as duplications, translocations, deletions and numerical anomalies such trisomy, monosomy (Caramazzaet al.,2010). Rearrangements of chromosome 1, especially the long arm, are frequently seen in hematologic malignancies. It usually occurs in advanced stages of malignancies and has been shown to correlate with poor prognosis. For example, multiple myeloma is one of the hematologic malignancies in which chromosome 1 anomalies are frequently seen. Deletions of short arm of chromosome 1 and amplification of long arm have been reported in MM (Marzin et al.,2006). It is stated that especially the long arm reorganizations provide the advantage of selection to cells during tumor development. (Sawyer, 2011).
In this study, retrospective analyses of 35 hematological patients with various chromosome 1 anomalies were done. Those were selected among the patients having cytogenetic analysis performed in our center between 2008 and 2016. The chromosomal anomalies and clinical features of those patients were correlated. Moreover, the effect of chromosome 1 anomalies to the prognosis of those patients was discussed.

## Materials and methods

Thirty five adult hematology patients with various chromosome 1 anomalies were included in this study. Conventional cytogenetic analysis of those patients was performed by investigating metaphases of 24 hours stimulated bone marrow samples. In this method, firstly patients' bone marrow samples were incubated at $37^0$C after adding uridine and 5-fluoro 2-deoxiuridine. After overnight incubation thymidine is added. Later, 30 minutes incubation in colsemide (0.05 g/mL) was done. Afterwards, the cells were put in hypotonic solution (0.075 M KCl) for 30 minutes and then they were fixed on slide with Carnoby solution (3 parts methanol/1 part iced acetic acid). After overnight incubation at $65^0$C, the samples were treated with trypsin and stained with Giemsa (GTG Banding). Analyses were performed on image analysis system (Metasystem, Germany). For each patient, 20 metaphases were analyzed and karyotypes written according to "2013 International Human Nomenclature System-ISCN" (Hafferet al.,2013).

## Results and Discussion

Chromosome 1 anomalies were determined in 35 patients (0.5 %) among 6865 patients having done their conventional bone marrow cytogenetic analysis in our center between January 2008 and March 2016. The ratio of chromosome 1 anomalies of totally 701 anomalies among 6865 patients was 4.9 %. Among total 35 patients with chromosome 1 anomaly, 22 (62.8 %) were men and 13 (37.1 %) were women. The average age of the patients was 57.5 and 60 years for men and women respectively.
Of the total of 35 patients with chromosome 1 anomaly, 11 were diagnosed with multiple myeloma (MM), 5 with acute myeloid leukemia, 7 with myelodysplastic syndrome (MDS), 2 with acute lymphoblastic leukemia, 3 with lymphoma (2 patients with B cell lymphoma and 1 patient with Burkitt lymphoma), 2 with chronic myelocytic leukemia (CML) and 1 patient with Polycytemiavera (PV), 1 with Aplastic anemia, 1 with Waldenstrom macroglobulinemia (WM), 1 with essential thrombocytosis and 1 with chronic myelomonocytic leukemia (CMML). In table 1, chromosome 1 anomalies according to the diagnosis are shown. Accordingly, del(1) was the most common anomaly in MM patients. MDS was the second most common diagnosis after MM in chromosome 1 anomaly patients, and in MDS patients derivative 1 anomaly was the most common. There were 5 AML patients, 2 patients had del(1) and others had rare translocations. In lymphoma patients again, del(1), derivation 1 and a very rare translocation were observed. In patients with PV, aplastic anemia and Waldenstrom macroglobunemia del(1) and derivative 1 anomalies were observed. The percentage distribution of chromosome 1 anomalies was shown in figure 1. Accordingly, the most common anomaly was deletion 1 which was seen in 16 (37%) of the patients. Second most common

**Table 1:** Chromosome 1 anomalies accordance to disease

| Disease | del(1) | t(1;V) | monosomy(1) | trisomy(1) | dup(1) | der(1) | i(1)(q10) |
|---|---|---|---|---|---|---|---|
| AML | 2 | 2 | - | - | - | 2 | - |
| MM | 9 | 1 | 1 | - | - | 3 | 1 |
| MDS | 1 | 1 | - | - | 1 | 4 | - |
| PV | - | - | - | - | - | 1 | - |
| BHL | 1 | - | - | - | - | 1 | - |
| BL | - | 1 | - | - | - | - | - |
| ALL | - | 1 | - | - | - | 1 | - |
| CML | 2 | 2 | - | 1 | - | - | - |
| Aplastic Anemia | 1 | - | - | - | - | - | - |
| Waldenstrom | - | - | - | - | - | 1 | - |
| ET | - | 1 | - | 1 | - | - | - |

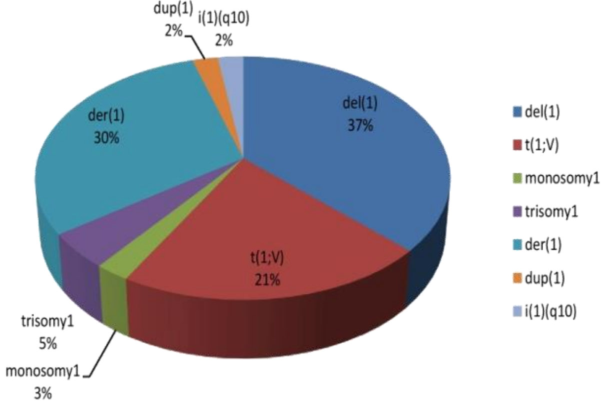
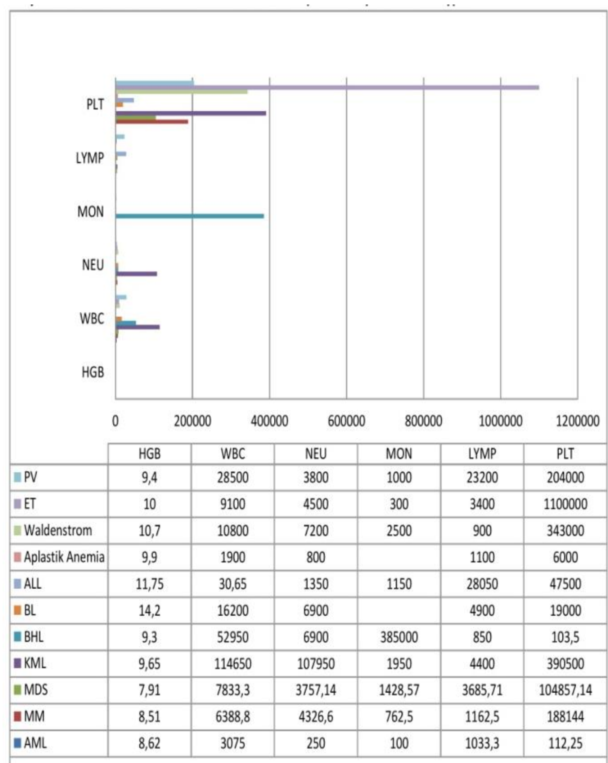



**Figure 1.** Distribution of chromosome 1 anomalies in 35 patients as a percentage

anomaly was derivation 1 which was seen in 13 (30%) of the patients. In our study group, we also detected translocations between chromosome 1 and other chromosomes, trisomy 1 (5%), monosomy 1 (3%), duplication 1 (2%) and isochromosome 1 (2%) were also detected. The age and sex distribution of the structural and numerical chromosome 1 anomalies is given in table 2. Accordingly, it can be said that all of the anomalies determined, the incidence in men was nearly two times higher than the incidence in women. The age interval of the patients with del(1), i(1)(q10) and t(1;V) was 48-55.The mean values of blood tests of the patients according to the disease groups is given in figure 2. Cytogenetic analysis is very important for hematologic patients. Besides being useful in diagnosis, it is also functional in determining subgroups of AML and MDS and in risk evaluation (Shumilovet al.,2018). In this retrospective study, chromosome 1 anomalies were determined most frequently in MM, MDS and AML patients. Karyotypes of the patients are listed in table 3. Overall, only in 3 (7%) patients numerical chromosome 1 anomalies were determined. Those patients with numeric anomalies also possessed some structural anomalies. The other 32 (93%) patients had only structural anomalies. When karyotypes of the patients are evaluated, among 35 patients with chromosome 1 anomalies, 25 (71.4%) patients had complex karyotypes and 10 patients (28.6 %) had non-complex karyotypes. Chromosome 1 anomalies are common in MM patients (Li et al., 2016), which was also observed in our study in 9 male and 2 female patients. All of those patients had complex karyotypes. In all but 3 MM patients with chromosome 1 anomalies, sex chromosome loss or excess was detected.

**Table 2:** Mean age and gender distributions of patients with structural and numerical chromosome 1 anomalies

| Anomaly | Age | Gender, Male: Female |
|---|---|---|
| del(1) | 48,2 | 11:5 |
| t(1;V) | 52,12 | 7:2 |
| der(1) | 55,4 | 8.5 |
| dup(1) | 23 | 1:0 |
| i(1)(q10) | 83 | 1:0 |
| monosomy(1) | 79 | 0:1 |
| trisomy(1) | | 0:1 |




| | HGB | WBC | NEU | MON | LYMP | PLT |
|---|---|---|---|---|---|---|
| PV | 9,4 | 28500 | 3800 | 1000 | 23200 | 204000 |
| ET | 10 | 9100 | 4500 | 300 | 3400 | 1100000 |
| Waldenstrom | 10,7 | 10800 | 7200 | 2500 | 900 | 343000 |
| Aplastik Anemia | 9,9 | 1900 | 800 | | 1100 | 6000 |
| ALL | 11,75 | 30,65 | 1350 | 1150 | 28050 | 47500 |
| BL | 14,2 | 16200 | 6900 | | 4900 | 19000 |
| BHL | 9,3 | 52950 | 6900 | 385000 | 850 | 103,5 |
| KML | 9,65 | 114650 | 107950 | 1950 | 4400 | 390500 |
| MDS | 7,91 | 7833,3 | 3757,14 | 1428,57 | 3685,71 | 104857,14 |
| MM | 8,51 | 6388,8 | 4326,6 | 762,5 | 1162,5 | 188144 |
| AML | 8,62 | 3075 | 250 | 100 | 1033,3 | 112,25 |

**Figure 2.** Distribution of blood values in patient population by disease subtype

Among MM patients, del(1) was the most common chromosome 1 anomalies detected in 9 patients. In 7 of those patients, deletion was on p arm of chromosome 1 and in 2 patients the deletion was on q arm. del(1p) is one of the characteristic chromosomal anomalies seen in MM patients (Szalatet al., 2015; Oi et.al., 2016; Josephet al., 2017). For example, Jung et al. (2018) detected 52 cases of del(1p) among 120 patients diagnosed with MM having cytogenetic anomalies. In our patient group, del(1p) was detected on p21, p13p36 and p31 regions, as it is stated in the literature (Manier et al., 2017). In 3 MM patients with del(1p) other chromosomal anomalies such as derivative 1 and monosomy 1 were also detected. In MM patients, del(1p) is associated with bad prognosis such as quick progress of disease and short survival time (Ouyanget et al., 2014; Hebraud et al., 2014). In our patient group, among 7 MM patients with del(1p) only 1 is still alive and the others passed away. The average survival time for those patients was 7 months after the diagnosis. Short survival time of MM patients with del(1p) in our group was compatible with the literature (Carballo-Zarate et al., 2017). In our study, second most common disease with chromosome 1 anomalies was MDS that affected 7 patients, 3 women and 4 men. In 5 patients with MDS and chromosome 1 anomalies the karyotypes were complex. The most commonly found chromosome 1 anomaly in 4 MDS patients was derivative 1. In all karyotypes with der(1), monosomy 5, monosomy 7 and trisomy 8 anomalies were also detected. In one of those patients del (1p) and the other patients very rare dic (1;15)(p11;p11) and dup(1)(q31q44) anomalies were found (Table 3).
All AML patients with chromosome 1 anomalies were men and among those 5 patients 3 had complex karyotypes and other 2 patients had non-complex karyotypes. In 2 AML patients del(1q), in one patient der(1), in 2 patients very rare t(1;11) and t(1;19) were determined. Again in 3 of those 5 patients, a monosomy 7 was found. One of the patients with derive 1 and the patient with del (1)(q32q43) are still alive.
Two of lymphoma patients with chromosome 1 anomalies had B-cell lymphoma, in those del(1)(p34p36) and der(21)t(1;21)(q21;q11.2) were determined respectively. The other patient with lymphoma was Burkitt's lymphoma and in this patient for the first time der(15)t(1;15)(q21;q11.2) and der(21)t(1;21)(q21;q11.2) translocations were determined. In the only patient in our study group diagnosed with Waldenstöm Macroglobunemia der(1) was determined. In the literature we can see that chromosome 1 anomalies are not present in Waldenstöm Macroglobunemia, instead 6q, 13q deletions and trisomy 18 chromosomal anomalies are seen (Kapoor et al., 2015; Hunter et al., 2017).
The last disease state and survivals of the patients with chromosome 1 anomalies is given on figure 3.

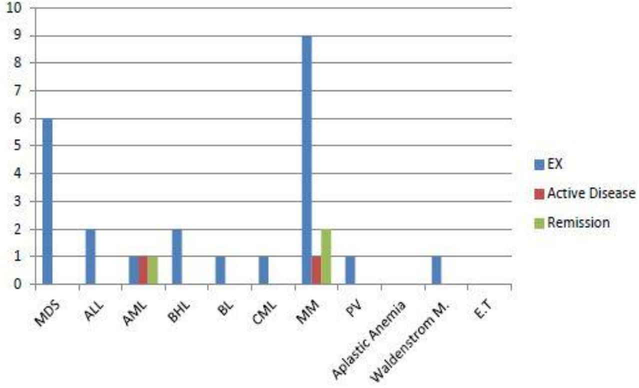



**Figure 3.** Last state disease by subtypes

**Table 3:** The karyotypes of the patients

| Patient Number | Diagnosis | Karyotypes |
|---|---|---|
| 1 | MM | 38-39,XY,+X,-Y,del(1)(p?),-4,-5,-5,-9,-13,-14,-16,-17,-18,-21,-22,+mar1,+mar2,+mar3[20]/70-76,XY[7]/46,XY[3] |
| 2 | MM | 45,XY,i(1)(q10),-2,-13,-14,del(16)(q24),+mar1,+mar2 [15]/45,XY,i(1)(q10),-2,-13,-14,-14,del(16)(q24),+mar2,+mar3 [10]/84-98<4n>XY,q(1)q(10),-2,-13,-13,-14,- |
| 3 | MM | 53,X,-Y,+del(1)(p35p21)x2,+der(2),+7,+der(14)x2,+15,-16,+18,+20,+21 [47]/46,XY [18] |
| 4 | MM | 49-55,XX,+X,del(1)(p31),-1,-4,+5,-6,+7,+8,-11,+12,-12,-13,-14,+17,-17,+18,+19,+20,+21,+22,+ma1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7,+mar8 [52]/46,XX [38] |
| 5 | MM | 50,X,-X,+del(1)(p35p21),+5,+5,-11,-16,+19,+mar1,+mar2,+mar3 [8]/46,XX[52] |
| 6 | MM | 90,XY,+X,der(1)x2,del(1)(p13p16)x4,+2,+3,+3,+5,+6,+7,+8,+8,+8,+9,+10,+10,+10,+11,+12,+13,+13,+14,+15,+16,+16,+16,+16,+17,+18,+19,+19,+20,+20,+20,+21,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7[2],46,XY[18] |
| 7 | MM | 46,XY,del(1)(q31q36),-12,-13,-17,+19,+mar1,+mar2 [9]/46,XY[11] |
| 8 | MM | 44,X,-Y,del(1)(q32q43),-11,-14,-17,+mar1,+mar2[2]/46,XY[18] |
| 9 | MM | 44,X,-Y,del(1)(p13p35),+der(1),t(3;16)(q21;q22),-4,-13,-14,+mar1[8]/46,XY[42] |
| 10 | MM | 46,XY,dic(1;21)(p11p11),del(3)(q25q29),del(6)(q24q26),t(11;14)(q13;q32),del(13)(q14q21)[20] |
| 11 | MM | 44,XY,der(1),+del(1)(p13p36),t(9;9)(p22;p24),-13,der(14),-15,-16,-22,+mar1[17]/46,XY[3] |
| 12 | CML | 46,XY,t(1;2)(p36;p21)[20] |
| 13 | MDS | 45,XX,der(1),-4,-7,-12,+mar1,+mar2,+mar3[19]/46,XX[1] |
| 14 | MDS | 45,XY,+del(1)(p35;p21),-7,-16[47],46,XY [3] |
| 15 | MDS | 46,XY,dic(1;15)(p11;p11)[1]/46,XY,del(13)(q14q22)[21]/46,XY[28] |
| 16 | MDS | 46,XX,-5,-7,+8,del(12)(pter-p12),+mar1[21],43,XX,der(1),-2,-3,-5,-7,-9,-10,-12,-17,+mar1,+mar2,+mar3,+mar4,+mar5 [9]/46,XX[5] |
| 17 | MDS | 46,XY,dup(1)(q31q44)[20] |
| 18 | MDS | 44,XX,der(1),-5,-7,-9,der(17),+mar1[16]/46,XY[4] |
| 19 | MDS | 48,XY,+der(1),+8,del(20)(q10)[20] |
| 20 | AML | 46,X,-Y,del(1)(q32q43),del(2)(q33q37),-7,-8,-10,+17,+mar1,+mar2,+mar3,[13]/46,XY[7] |
| 21 | AML | 46,XY,del(1)(q31)[8]/46,XY [12] |
| 22 | AML | 45,XY,der(1),der(3),-7[16]/46,XY[4] |
| 23 | AML | 46,XY,t(1;11)(p31;q23),-4,-7,-9,-12,-13,-14,-17,-20,-21,-22,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7,+mar8,+mar9,+mar10[20] |
| 24 | AML | 46,XY,der(1),der(9)t(1;9)(q21;q34)[12]/47,sl,+6[8] |
| 25 | CML | 46,XX,del(1)(q31q42),-7,t(9;22)(q34;q11.2),-16,+mar1,+mar2[20] |
| 26 | CML | 47,XY,t(X;1)(q27-q28;p31-p32),+del(1)(p31-pter)[21]/46,+1,-6[25] |
| 27 | ALL | 46,XY,der(1)[12]/47,XY,+22,der(1)[8]/46,XY[20] |
| 28 | ALL | 46,XX,t(1;12)(p35;q24.1),t(9;22)(q34;q11.2)[27]/46,XX[13] |
| 29 | B-cell Lymphoma | 46,XY,del(1)(p34p36)[4]/46,XY,del(1)(p34p36),-14,+mar3[6]/46,XY,del(1)(p34p36),-14,+mar4[6],46,XY,del(1)(p34p36)-6,-20,+mar1,+mar2 [43] |
| 30 | B-cell Lymphoma | 47,XX,+X,der(1),der(3),-4,-7,-8,del(9)(p21),der(12),der(18),del(22)(q11.2),+mar1,+mar2,+mar3[22]/46,XX[48] |
| 31 | Waldenstrom | 46,XX,der(1)[8]/46,XX[42] |
| 32 | Aplastic anemia | 46,XX,del(1)(q42q43)[7]/46,XX[13] |
| 33 | ET | 46,XX,+1,t(1;15)(p10;p10)[24]/46,XX[26] |
| 34 | P.V | 46,XY,der(1),der(10),+15,-18[4] 47,XY,der(1),der(6)x2,-10,+18,+mar1[7]/46,XY [39] |
| 35 | Burkitt Lymphoma | 46,XX,der(15),t(1;15)(q21;q11.2),der(21),t(1;21)(q21;q11.2),t(8;14)(q24;q32)[20] |

The data about survival of 5 patients were not given because these patients were out of follow up. The other 30 patients, except 2 AML and 3 MM patients, have passed away.

In 9 out of 35 patients of our study group, the translocation of chromosome 1 to other chromosomes was detected. Among those, 5 were the translocation that are not reported in the databases for the associated breaking points (Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2017; Mitelman et al., 2017). One of them was t(X;1)(q27-q28;p31-p32) translocation which was seen our 87 old male patient with CML. In the karyotype of the this patient, we also detected del(1)(p31-pter) and trisomy 1. The other translocation that is reported here for the first time is t(1;12)(p35;q24.1). This translocation was detected in one of the patient who diagnosed as Philadelphia chromosome positive ALL. In the databases searched (Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2017; Mitelman et al., 2017) the translocations between 1p and 12q were reported but breaking points between p35 and q24.1 have not been reported before. Another anomaly that was described for the first time was dic (1;21)(p11;p11). There is no previous report for a translocation between chromosomes 1 and 21 at those breaking points. Therefore, we here describe this translocation in a MM patient for the first time. The patient with dic (1;21)(p11;p11) had also t(11;14)(q13;q32) which is common in MM patients and it is a good prognosis marker (Kumar et al.,2018), also del(13)(q14q21) was found in that patient and this translocation was linked with short survival time. Therefore it is not possible to directly link the effect on dic (1;21)(p11;p11) with the prognosis of the disease. In a female patient 2 new translocations were determined.

Those translocations were der(15)t(1;15)(q21;q11.2) and der(21)t(1;21)(q21;q11.2) and they were found together with t(8;14)(q24;q32) which is characteristic for Burkitt lymphoma.

As a result of having der(15)t(1;15)(q21;q11.2) and der(21)t(1;21)(q21;q11.2) in her karyotype, the patient had tetrasomy on (1q). Siddiki et al.(2018) stated that t(8;14)(q24;q32) seen together with tetrasomy (1q) could be a marker for very bad prognosis Accordingly in our case, the patient with these anomalies died 8 months after the diagnosis.

The translocations found in some of our patients were very rare translocations. One of those was t(1;2)(p36;p21) which was determined in a male patient with chronic myelomonocytic leukemia (CMML). So far, there are only 9 hematology patients reported to have this translocation and all of them had some other disease besides CMML (Storlazzi et al., 2008; Mitelman et al., 2019). The patient died 4 years after the diagnosis and there is no definitive information about the effect of those anomalies on the disease prognosis. t(1;15)(p10p10) was detected in a 80 years old patient with essential thrombositopenia (ET).This translocation was reported only in one case so far with breast cancer in a complex karyotype (Adeyinka et al., 1999) and this is the first report of this anomaly in an ET patient. The genes related to this translocation are still unknown. t(1;11)(p22;q23) were determined in one adult patient with AML (Figure 4). MLL gene located on 11q23 codes for a methyl transferase active in epigenetic modification of histon proteins and 11q23 anomalies can be seen in patients with myeloid malignancies (Zhao et al., 2014).

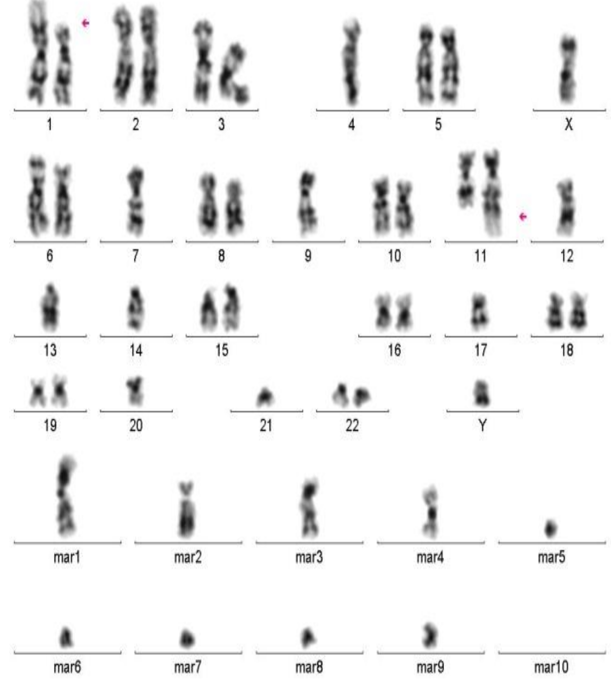



**Figure 4.** t(1;11)(p22;q23) in one adult patient with AML

## Conclusion

Genetic aberrations in the form of chromosomal anomalies are frequent cause of hematologic malignancies. The effect on disease pathogenesis and prognosis of some of those anomalies is known and some needs to be investigated and determined. The results of our research indicate that the determination of effect on disease pathogenesis of newly defined and rare chromosomal anomalies could be helpful on increasing the treatment options of many hematologic malignant diseases.

## Conflict of interest

Authors declare that there is no conflict of interest.

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*Research article*

# CHROMOSOME STABILITY OF *IN VITRO* PROPAGATED C*ucurbita* CULTIVARS

Buse Dursun[1], Ahu Altınkut Uncuoğlu[2], Yıldız Aydin[1*]

[1]*Marmara University, Faculty of Science and Arts, Department of Biology, Göztepe Campus, Istanbul, Turkey*
[2]*Marmara University, Faculty of Engineering, Department of Bioengineering, Göztepe Campus, Istanbul, Turkey*

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***Correspondence**
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ayildiz@marmara.edu.tr
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*Cucurbita pepo L., in vitro micropropagation, karyotype analysis*

## Abstract

*Cucurbita pepo* L., a member of Cucurbitaceae family, is an annual plant with herbaceous stems, broad leaves and superficial scattered roots. Monoecious flower structure in the Cucurbitaceae family and the differences in the maturing time of male and female organs in flowers cause an increase in the foreign fertilization rate. Therefore, there may be positive or negative changes in the existing characteristics of the species. Micropropagation method can be performed in pumpkin species for clonal propagation, but their genetic stability in tissue culture is an important consideration. Chromosome number and morphology are primary cytogenetic parameters that must remain stable after *in vitro* propagation. We preformed cytogenetic analysis of different hybrid pumpkin genotypes cultivated in our country (Ardendo, Angelina, Torpido, Roni, Sena Hanım) in order to determinate their chromosome stability level. Cotyledon nodes, nodes, shoot apex, hypocotyl and internode explants were prepared from the 4-week old *C. pepo* seedlings by making a horizontal slice through the hypocotyl region. The highest shoot and callus regeneration was obtained in Torpido genotypes in cotyledonary node explants produced multiple shoots placed in tissue culture media MS+1 mg/l N6-benzylamino-purine BA. The chromosome number and karyotype analysis were determined in control and *in vitro* propagated *Cucurbita pepo* L. plants and ploidy levels were confirmed to be 2n = 40.

## Introduction

The Cucurbitaceae family, also referred as cucurbits, forms a very large group with approximately 130 genera and 800 species. Cucurbits plants can be cultivated worldwide in warmer region and are popular food crop plants. Some of these species include squashes, pumpkins, melons and gourds (Perez Gutierrez, 2016). The cultivated *Cucurbita* species are: *C. argyrosperma, C. maxima, C.*

*moschata*, and *C. pepo*, which include both summer squash and winter squash as well as ornamental gourds. The most important species in terms of agricultural production worldwide are *C. maxima, C. moschata* and *C. pepo*. Turkey is one of the world's leading cucurbit producers by taking the 7th place in cucurbit production in the world (Turkish Statistical Institute, 2018 http://www.tuik.gov.tr). The most commonly grown species in Turkey are *Cucurbita pepo* L., *Cucurbita maxima* Duch. and *Cucurbita moschata* Pour. including summer squash and winter squash. (Güner et al., 2012). *Cucurbita pepo* L. was found in summer grown squashes while *Cucurbita maxima* Duch. and *Cucurbita moschata* Pour. among the winter grown squashes. Among these species *C. pepo* is the most commonly cultivated pumpkin (Sunulu & Yağcıoğlu, 2014). The leaves of *C. pepo* contain 43.8% protein (Oloyede, 2012), the fruits are characterized by low fat content (2.3%), high carbohydrates (66%) and low proteins (3%) and very high carotenoids contents (171.9 to 461.9 µg/g) (Adedayo et al., 2013). Seeds are used as an antidiabetic, antihypertensive, antitumor, antimutagenic, immunomodulatory, antibacterial, anti-hypercholesterolemic, intestinal antiparasitic, antalgic, and anti-inflammatory agent (Kostalova et al., 2009). *C. pepo* has high agricultural properties and it is included in the fruit-eaten vegetables class. Its fruits can be used in food and cosmetic industry while seeds are used as snack foods (Sağlam & Çetin, 2018).

Development of *in vitro* regeneration technology is one of the solutions for crop improvement (Kurozawa et al., 1997; Ananthakrishnan et al., 2003). Since the first experiments of Gottlieb Haberlandt in the early 1900 on the *in vitro* cultivation of plant tissue, the fields of application have expanded from research of plant physiology to applications in breeding, molecular and microbiology and it became also an important tool for commercial plant production (Laimer & Rücker, 2003). Plant regeneration via shoot organogenesis is a more suitable and rapid approach (Obembe et al., 2011) in comparison to traditional *in situ* cultivation. Plant population produced by direct organogenesis from shoot meristem and leaf explants are homogenous. Therefore, genetically identical plants could be provided via regeneration in large scales (Seyis et al., 2017). Some factors such as Plant Growth Regulator (PGR) balance, culture conditions, genotype and explant type are important for successful plant regeneration. Ananthakrishnan et al. (2003) reported the regeneration of *C. pepo* from seedling-derived cotyledon explant through direct organogenesis. Schroeder (1968) documented the regeneration of zucchini squash (*C. pepo*) from flesh pericarp wall-derived callus through somatic embryogenesis. Carol et al. (1995) in their work reported the initiation of somatic embryos via cotyledon explant in six squash cultivars (*C. pepo*).

Tissue culture techniques, organogenesis and micropropagation enable clonal production by performing *in vitro* regeneration.

This clonal production enables the stability of the desired ploidy levels in plant species. Lee et al. (2003) reported that they obtained 82 and 92% shoot regeneration ratio in *C. maxima* for two cultivars using cotyledon explants through effective plant regeneration protocol via organogenesis. Their flow cytometric analysis revealed that most of the regenerated plants were diploid (45.8-95.0%), tetraploid (0-4.2%) and mixoploid (5.0-50.0%) using different BA concentrations and cotyledon sizes Obembe et al. (2017) cultured hypocotyl, cotyledonary node and cotyledon explants derived from 4-week old seedlings on MS medium fortified with 0.00, 1.00, 2.00 and 3.00 mg/l of BAP in combination with 0.00 or 0.05 mg/l of 2,4-D and investigated for callus, shoot and root induction. They reported that when the different explant types were cultured on MS media amended with the different concentrations of BAP in combination with 2,4-D, neither shoot nor root induction was observed.

The aim of the present study was to develop a highly repetitive protocol for the *in vitro* regeneration of an indigenous Turkish pumpkin from seedling derived different explants and to determine their chromosome stability.

## Material and methods

Different hybrid squash varieties (Ardendo, Angelina, Sena Hanım, Roni, Torpido) seeds (2n=2x= 40) were obtained from Thrace Agricultural Research Institute (TARI).

*In vitro regeneration*

Seed surface sterilization were performed by immersing seeds in 70% ethanol for 3 min followed by treating with 15% commercial bleaching solution for 15 min. Rinse (each 5 min) in sterile distilled water was repeated for three time (Kurtar et al., 1999). Sterilized seeds were transferred to magenta (tissue culture container) containing pure ½MS media (Murashige & Skoog, 1962) containing 30 g/l sucrose and 1,1 g/l gelrite. pH value of all media was adjusted to 5.8 before autoclaving.

Each magenta cap was wrapped with aluminum folia and incubed in a growth chamber programmed with an 16/8 h light/dark cycle and 25±2 °C temperature. Explants (node, cotyledon node, shoot apex and internode) were obtained from four weeks old plantlets. Explants were transferred in tissue culture media containing 1, 2, and 3 mg/l BA for shoot and callus regeneration (Obembe, 2017) and regularly (every 4 weeks) subcultured on the same media. Developed shoots were transferred in tissue culture media containing 1 mg/l indole-3-butyric acid (IBA) or plant growth regulator free MS medium (Ananthakrishnan et al., 2003) for rooting. All cultures were incubated in a growth chamber at 25°C and 6000 lux fluorescent light under a 16/8-h photoperiod. Successfully rooted shoots were planted into the pots containing soil for acclimatization and plants were gradually acclimatized to dry air to keep the environment moist. All micropropagation studies were performed in 3 replicates and 3 subcultures were performed for each replicate.

The percentage of explants on which buds developed and the number of distinguishable shoot buds on each explant were recorded. Tukey test was performed by using single factor analysis of variance (ANOVA) for all micropropagation studies.

*Cytogenetic analysis*

Representative root samples (obtained from apical buds only) for all plant propagation categories h) were excised when they were about 1 cm in length. Pretreatment with distilled water at 4°C for 12 h was followed with root tips fixation in acetic acid: ethanol (1:3 v/v) solution for 1−2 days. The samples were then hydrolyzed in 1 N HCl for 5 min at 60°C. Afterward, squash mounts were prepared with Feulgen in order to determine chromosome number and the ploidy levels (Metwally et al. 1998).

## Results and Discussion

*In vitro regeneration*

Seeds of Ardendo, Roni, Sena Hanım, Torpido and Angelina genotypes were sterilized under sterile conditions and planted in MS medium. Seeds were kept in the dark for 24 hours and germination percentages were calculated after four weeks. The highest germination percentage (100%) was achieved in the Torpedo genotype. It has been well known that genotype, explants type and composition of the medium are important factors on organogenesis. Furthermore, the physiological conditions and hormonal contents of explants are crucial points for regeneration potential. To establish optimal conditions for adventitious shoot induction, a variety of explants (cotyledon node, shoot apex and node) were prepared from seedlings and were cultured using media containing different concentrations of BA (1mg/l (K1), 2mg/l (K2), 3mg/l (K3). Shoot regeneration was obtained from cotyledon node, shoot apex and node explants in all genotypes (Ardendo, Angelina, Roni, Sena Hanım, Torpido) and in all media (Figure 1). Shoot regeneration was not achieved in hypocotyl and internode explants. Callus regeneration was obtained in all genotypes and all explants (Cotyledon node, shoot tip, node, internode and hypocotyl). Hypocotyl and internode explants showed low callus regeneration in all genotypes compared to cotyledon node, shoot apex and node explants. In the hypocotyl explants, no regeneration was observed with callus formation on the parts of the explants touching the medium.

According to the data obtained, the highest shoot regeneration response (92.98%) was achieved in the cotyledon node explant of Ardendo genotype. (Figure 2). The highest callus regeneration response (92.18%) was achieved in the node explant of the Roni genotype (Figure 3). Under proper culture conditions, plant cells possess a capacity to regenerate organs from specialized somatic tissues through a process known as de novo organogenesis. The importance of genotype for shoot and callus regeneration was emphasized in different studies.

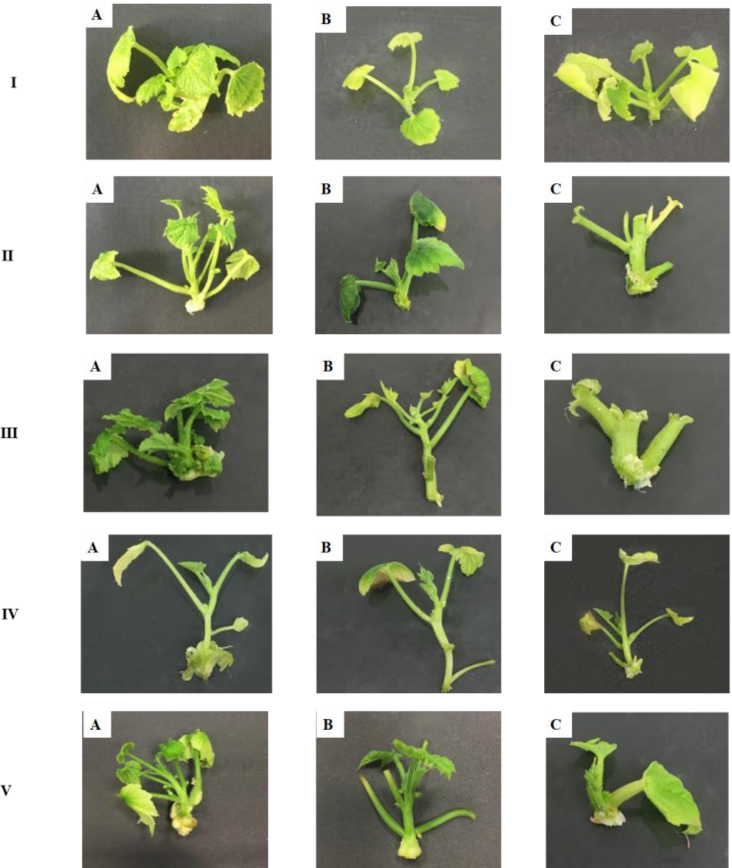


**Figure 1.** Shoot multiplication with non-significant callus formation in MS media supplemented with BA (2 mg/l) from cotyledone nod, shoot apex and nod segment in Angelina (I), Ardendo (II), Roni (III), Sena Hanım (IV), Torpedo (V) genotypes.

A shoot regeneration protocol was developed for five cultivars of the Cucurbitaceae by Abrie & Van Staden (2001). They tested the effects of combinations of BA, kinetin, iP and TDZ with IAA in the culture medium on shoot regeneration of cotyledonary explants and showed that the cultivars *Cucurbita maxima* cv. A-line, *C. maxima* cv. Chicago Warted and *C. pepo* cv. Rolet, did not form shoots on any of the treatments. Although *Cucumis sativus* cv. Ashley responded poorly shoot development, *Cucumis melo* L. cv. Hales Best 36 variety regenerated successfully. In our case the Torpido genotype showed higher regeneration capacity compared with other genotypes. Cotyledons are frequently used as explants in shoot and callus regeneration in Cucurbitaceae family (Ananthakrishnan et al., 2003; Lee et al., 2003; Han et al., 2004; Kim et al., 2010; Obembe et al., 2017). After cotyledons, hypocotyls (Pal et al., 2007; Obembe et al., 2017) and shoot tips (Sarowar et al., 2003) are also preferably used as explants. It was observed that the highest shoot and callus regeneration rates were in cotyledon node explant (Obembe et al., 2017). However, in a few studies the

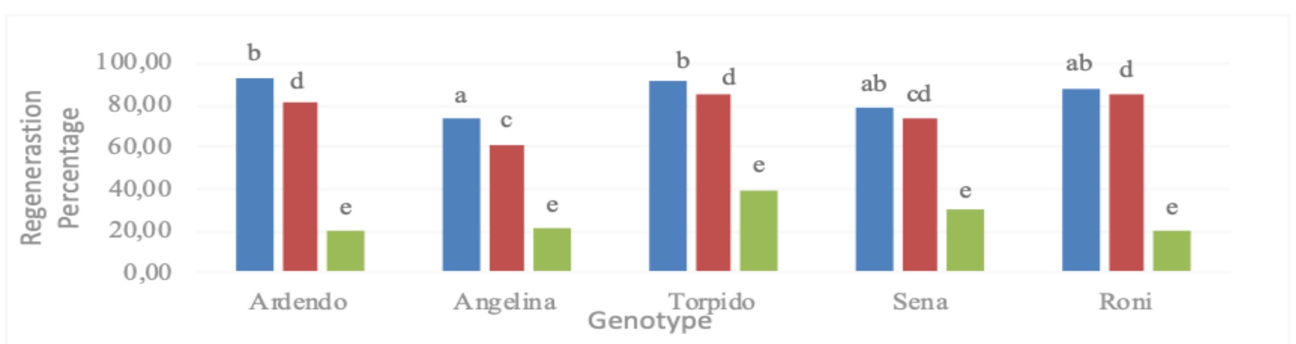


**Figure 2.** Shoot regeneration rates in MS media supplemented with 1mg/l (K1), 2mg/l (K2), 3mg/l (K3) BA from cotyledone nod, shoot apex and nod segment in all tested genotypes (three trials and three subculture results)

regeneration rate from the hypocotyl explant was reported to be higher than the regeneration rate from the cotyledon explant (Pal et al., 2007). Obembe et al. (2017) did not achieved shoot regeneration from the hypocotyl explants. In this study, the highest shoot regeneration rate, in all genotypes, were obtained from cotyledon nodes and shoot apex. Shoot regeneration was not obtained from hypocotyl and internode explants. Therefore, our findings are in concordance with other studies.

It is observed that cytokinins (BA, IP, Kinetin, Zeatin) and auxins (IAA, NAA, 2,4-D) are used alone or in combination for shoot and callus regeneration in Cucurbitaceae family (Abrie & Van Staden, 2001; Ananthakrishnan et al., 2003; Lee et al., 2003; Sarowar et al., 2003). According to these studies, BA has been reported to be more efficient in callus and shoot regeneration than other cytokinins (Abrie & Van Staden, 2001). Obembe et al. (2017) reported that a combination of 1.00 mg/l BAP with 0.05 mg/l 2,4-D was optimum for callus induction from hypocotyl and cotyledonary node explants, while for cotyledon explants, 2.00 mg/l BAP in combination with 0.05 mg/l 2,4-D was preferred. Cotyledonary node explants and cotyledonary node explant-derived callus responded with multiple shoots on full strength Murashige and Skoog (MS) medium (control) devoid of Plant Growth Regulators (PGRs). In this study, different concentrations of BA (1 mg/l, 2 mg/l BA, 3 mg/l) were found to decrease in stem and callus regeneration as the BA concentration increased. The optimal concentration for shoot regeneration was 1 mg/l BA.

*Root Regeneration*

For root regeneration, basal MS medium and MS medium containing 1 mg/L IBA were used. Only regenerated shoots from the shoot apex and cotyledon node explants was used for rhizogenesis. Root regeneration was not achieved in the nod explants. The highest rooting rate (83.28%) in regenerated shoots was obtained in the Torpedo genotype. The root regeneration rate was 90.02% in basal MS medium and 49.63% in MS medium supplemented with 1 mg/L IBA.

In study conducted by Lee et al. (2003), regenerated cotyledons were successfully rooted in basal MS medium. Sarover et al. (2003), used the shoot apex in their study and regenerated shoots successfully rooted in MS medium containing 1 mg/ml IBA. In this study, cotyledon nodes and shoot apex were successfully rooted in MS medium containing 1 mg/ml IBA or without plant growth regulator. MS medium containing 1 mg/ml IBA or MS medium without plant growth regulator were used in different studies (Lee et al., 2003; Sarowar et al., 2003; Han et al., 2004; Pal et al. 2007; Mookhan, 2015) for rooting. On the other hand, Kim et al. (2010), preferred MS medium containing 0.1mg/l NAA for rooting. In our study on basal MS higher root regeneration was observed than on MS medium containing 1mg/l IBA and this result is compatible with recent findings (Obembe et al. 2017).

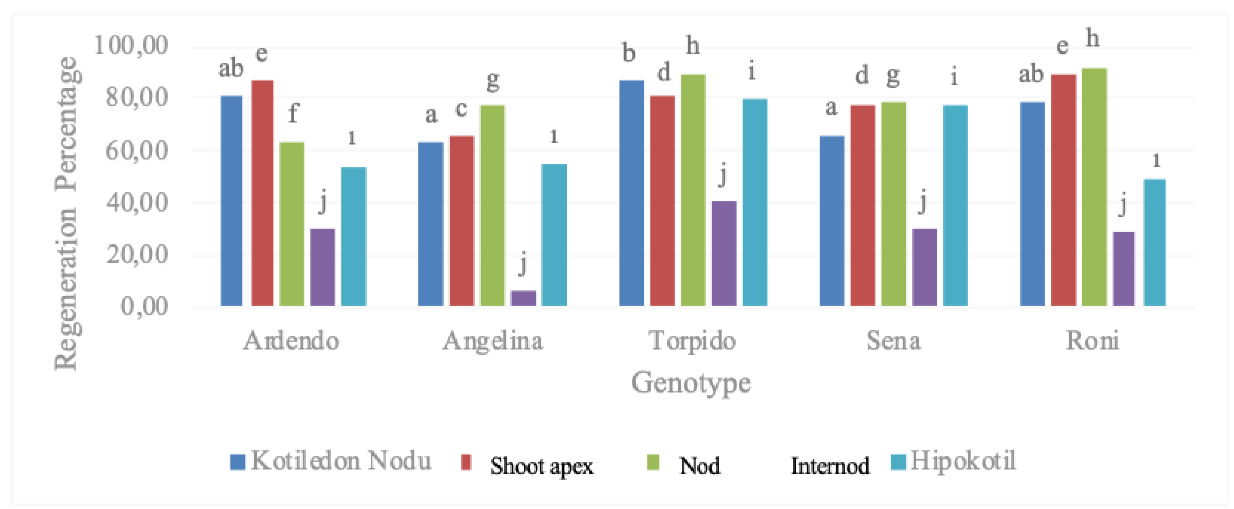


**Figure 3.** Callus regeneration rates of different explants from Ardendo, Angelina, Sena Hanım, Roni, Torpedo genotypes in MS medium containing 1mg/l (K1), 2mg/l (K2), 3mg/l (K3) BA (three trials and three subculture results)

*Acclimatization*

Regenerated shoots with healthy roots were planted in pots containing 1 kg of sterile soil. The plants in pots were covered with stretch film to keep the environment moist and the plants were gradually acclimated to dry air. The highest acclimatization percentage was achieved from the shoot apex explant of the Roni genotype with 98.88% acclimatized plants. In general, plants obtained from cotyledon nodes were able to survive in soil better than plants obtained from shoot apex.

*Ploidy Analysis*

Determination of ploidy levels in regenerated *C. pepo* plants was achieved by chromosome counting in root tips of one-week old seedlings. Chromosome staining was obtained by Feulgen reaction and 2n = 40 chromosomes were successfully counted in all genotypes by karyotype and idiogram analysis. The germinated seedlings control and the root tips taken from MS medium without plant growth regulator as a result of 3 subcultures in K1 (1 mg/l BA) medium. In both applications, 2n = 40 chromosomes were successfully counted. This emphasizes that the ploidy levels do not change after the root and shoot regeneration in Ardendo genotype and that the study is reliable (Figure 4).

The fact that the *Cucurbita* chromosomes are very small in the mitotic stage and that they do not show a good distribution in the cell, makes *Cucurbita* karyotype analysis quite difficult (Whitaker, 1930; Whitaker & Davis, 1962). Although it was previously stated that the number of chromosomes in *C. pepo* was 2n = 48, it is now known that the number of chromosomes in *C. pepo* is 2n = 40 (Whitaker, 1930; Tatum et al. 2006). In tissue culture studies, there is always a possibility of difference in ploidy levels in the control and regenerated plants. Lee et al. (2003) found that 73.7% of the regenerated plants were diploid, 25.8% were mixoploid and 0.5% were tetraploid. In another study, it was reported that all regenerated squash species are diploid (Ananthakrishnan et al., 2003). In this study, although small chromosomes in all pumpkin genotypes made karyotype analysis difficult, 2n = 40 chromosomes were successfully counted and no changes in ploidy level in the control and *in vitro* regenerated plants.

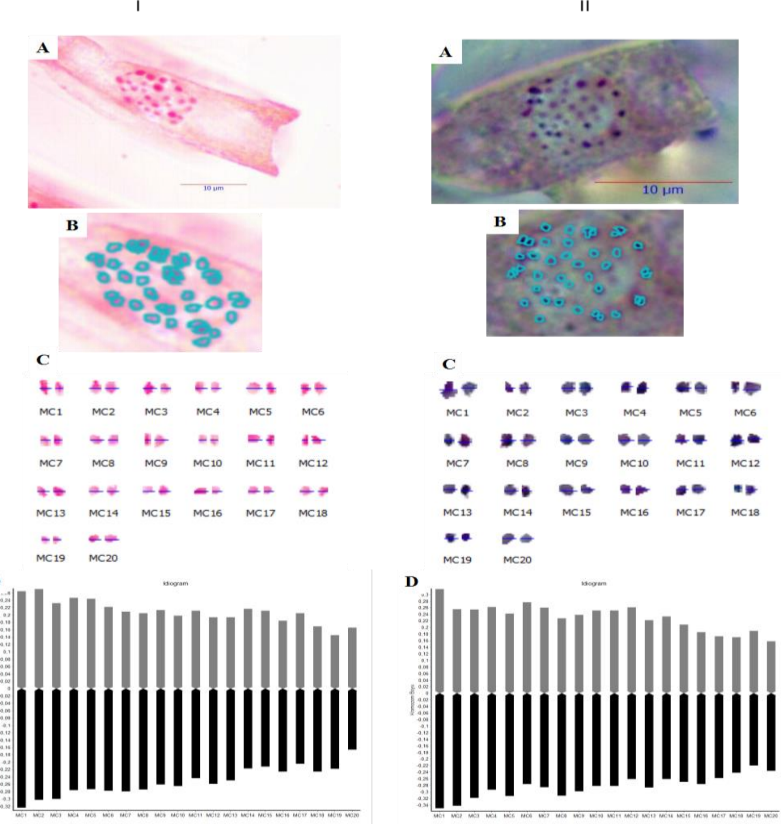


**Figure 4.** Analysis of the Ardendo genotype in the control group (I), 3 subculture results of Ardendo genotype in K1 (1 mg/l BA) medium (II). A) Chromosome photograph; B) Chromosome counting; C) Karyotype analysis; D) Idiogram

## Conclusion

Although, different types of explants have been using by different researchers, it is clear that the influence of the genotype is often the parameter which will determine how well a cultivar will react to growth regulators in tissue culture studies. Therefore, it is necessary to test the response of specific explants from each cultivar to different levels of growth regulators to determine optimum culture conditions for shoot regeneration.

The success of *in vitro* culture depends mainly on the growth conditions of the source material, medium composition, culture conditions and on the genotypes of donor plants (Tiwari et al., 2013). In our case the Torpedo genotype showed higher regeneration capacity compared with the other. The most successful explant type for shoot and callus regeneration was the cotyledon node and the most successful culture medium for shoot and callus regeneration was K1 (MS + 1 mg/l BA). The best root regeneration was achieved in the torpedo genotype, cotyledon node giving the best root regeneration and MS growth medium without plant growth regulators was became the most successful nutrient medium for root regeneration. We established an *in vitro* propagation method for *Cucurbita* cultivars by culturing the meristem in MS medium containing different concentrations of BA. The resulting meristem-derived plants were cytogenetically stable, therefore, we expect that the *in vitro* propagation method implementing 1 mg/l BA will be applicable in commercial production of *Cucurbita* plants.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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*Research article*

# RELATIONSHIP BETWEEN *H1* AND *H2* HAPLOTYPES OF THE 17Q21 INVERSION AND PREGNANCY LOSS IN BOSNIAN POPULATION: A CASE - CONTROL STUDY

Grazyna Adler[1*], Emir Mahmutbegović[2], Izabela Uzar[3], Mateusz Adler[4], Nevena Mahmutbegović[5]

[1]*Department of Studies in Antropogenetics and Biogerontology, Pomeranian Medical University, 71-210 Szczecin, Zolnierska 48, Poland*
[2]*Institution of Health Protection of Women and Motherhood Canton Sarajevo, Sarajevo, Bosnia and Herzegovina*
[3]*Department of General Pharmacology and Pharmacoeconomics, Pomeranian Medical University, 71-252 Szczecin, Poland*
[4]*Warsaw School of Economics, 02-554 Warsaw, Poland*
[5]*Neurology Clinic, Clinical Center of University of Sarajevo, Sarajevo, Bosnia and Herzegovina*

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***Correspondence**
E-mail: gra2@op.pl
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*17q21 inversion, H1 and H2 haplotypes, pregnancy loss, number of children*

## Abstract

The 17q21.31 band is one of the most structurally complex and evolutionary dynamic regions of the genome. Frequencies of two single nucleotide polymorphisms (SNPs): rs9468 and rs1800547 determine worldwide distribution of *H1* and *H2* haplotypes. Recent studies have demonstrated that *H2* haplotype is ancestral in hominoids and under positive selection in European population. The role of non-inverted orientation (*H1* haplotype) and inverted orientation (*H2*) remains unclear, where it is suggested that mothers who are *H1H2* heterozygotes on average tend to have more children than *H2H2* homozygotes.We investigated the prevalence of the inverted 17q21 haplotype in 154 women with pregnancy loss and 154 mothers with at last one live-born child, mean age: 33.0 (±5.4) y/o and 31.4 (±6.7) y/o. All 308 women were of Bosnian origin from Sarajevo. Following DNA extraction from buccal swabs, the genotyping was performed. For statistical analysis R CRAN software was used. Haplotypes distribution was compared between groups. In women with and without pregnancy loss we identified: 74.7% and 79.2% *H1H1*, 24.0% and 17.5% *H1H2* and 1.3% and 3.3% *H2H2* haplotypes. There were no significant differences between the distributions of haplotypes in women with and without pregnancy loss. Statistically significant difference between the average number of children in women with *H1H2* haplotype ($n_{avg.}$ = 1.54) in comparison to women with *H2H2* haplotype ($n_{avg.}$ = 1.29), was not found. Haplotype *H2* of the 17q21.31 inversion was not linked to pregnancy loss and number of children in Bosnian women.

## Introduction

Recurrent pregnancy loss is a pregnancy complication characterized by two or more consecutive spontaneous abortions which affects 1 to 3% of fertile couples (Li et al., 2016). According to the previous reports, pregnancy loss (PL) may have multifactorial background: environmental, anatomical, immune, as well as endocrine or genetic. However, despite the awareness of the impact of the above-mentioned factors, approximately 50% of pregnancy loss cases still remain unexplained (Musters et al., 2011). Development of molecular techniques over the past decades resulted in elucidating the role of genetic risk factors in susceptibility to different conditions including pregnancy loss.

Inversions – one of genetic factors – could be associated with phenotypic effects of clinical importance and have important consequences in humans (Puig et al., 2015; Giner-Delgado et al., 2019). One of them is the approximately 900 kb chromosome inversion polymorphism at the 17q21.31 arising about 3 million years ago, including among others the *MAPT* (microtubule-associated protein tau) gene (GenBank: NC_000017.11, Gene ID: 4137), which defines two distinct lineages, *H1* and *H2* (Stefansson et al., 2005). Worldwide distribution of *H1* and *H2* haplotypes is linked to frequencies of two single nucleotide polymorphisms: rs9468 and rs1800547. The rs1800547A and rs9468T alleles are on *H1* haplotype background, and the G and C alleles are on *H2* background.

The role of non-inverted orientation (*H1* haplotype) and inverted orientation (*H2*) has not yet been fully elucidated. Some authors reported that the 17q21.31 inversion can be considered as a potential biomarker of Alzheimer's and Parkinson's diseases as well as mental disorders (Rao et al., 2010; Spencer et al., 2011; Okbay et al., 2016; Babic Leko et al., 2018). The inversion status has also shown to affect the expression of several genes in the 17q21.31 region (de Jong S et al., 2012). Analysis of the 17q21.31 inversion region demonstrated that *H2* haplotype is ancestral in hominoids (Stefansson et al., 2005). In Europeans, the inversion seems to have been favoured by natural selection in the past $1{\cdot}10^3$ years and the distribution of *H2* haplotype varies in different populations. It ranges from 4.3% in Finns, 15.0% in Danes through 17.7% in the Irishs, 18.9% in French, 23.9% in Hungarians to 27.7% in Greeks and 37.5% in Sardinians (Donnelly et al., 2010). Decreasing gradient from south to north Europe is observed. In Africans this haplotype is rare (probably as a result of European admixture) while in Asians is almost absent (Donnelly et al., 2010).

A 900-kb inversion polymorphism at 17q21.31 with two haplotypes (*H1* and *H2*) was first described over a decade ago (Stefansson et al., 2005). Authors have suggested that *H1H2* heterozygotes women from Iceland, on average, have more children than *H2H2* homozygotes. Additionally, heterozygotes have higher recombination rates (Stefansson et al., 2005).

The aim of our study was to establish the distribution of allele and genotype frequencies of the 17q21.31 inversion in women with PL and mothers with at last one live-born child from the general population of Sarajevo, Bosnia and Herzegovina (B&H). We also compared number of children in women that are *H1H2* heterozygotes and women that are *H2H2* homozygotes.

## Material and methods

The present study was conducted in accordance with the standards of the Declaration of Helsinki (1975, revised 2000), and the protocol of the study was approved by the local Ethics Committees (decision ref. numbers: B&H: 10-1285-03-14; Poland: KB-0012/38/13 and KB-0012/119/18). Informed consent was obtained from all individual participants included in the study. According to the definition of ASRM (American Society of Reproductive Medicine) PL is spontaneous abortion of an embryo or fetus before the 20th week of pregnancy or when the fetus weighs less than 500 grams or measures less than 25 cm while RPL (recurrent pregnancy loss) refers to two or more failed pregnancies (Practice Committee of the American Society for Reproductive Medicine, 2008).

### *Subjects*

All women were recruited from Institution of Health Protection of Women and Motherhood in Sarajevo, B&H. Twins and women with any serious illness

**Table 1.** Characteristics of group of women with pregnancy loss

| | 1st trim. n= 118 | 2nd trim. n=21 | 1st+2nd trim.* n=15 |
|---|---|---|---|
| Age (years±SD) | 32.8 (±5.4) | 33.5 (±4.7) | 33.3 (±3.7) |
| Weight (kg) | 74.3 (±9.5) | 72.2 (±9.8) | 73.1 (±6.7) |
| No. of pregnancy loss, (average/range) | 159 (1.3/1-4) | 30 (1.4/1-3) | 31 (2.1/1-3) |
| The average week of pregnancy loss, average (±SD) | 9.1 (±1.6) | 18.1 (±3.7) | 13.5 (5.6) |
| Min. week of miscarriage | 6 | 13 | 13 |
| Max. week of miscarriage | 12 | 24 | 28 |
| No. of successful pregnancies, (average/ range) | 172 (1.5/0-5) | 24 (1.1/0-4) | 18 (1.2/0-5) |

*women with both first and second trimester losses

(including hepatic, pulmonary, renal disorders and cancer) were excluded from the study. For the purpose of the study, we chose 154 women with pregnancy loss (PL) and 154 mothers with at last one live-born child as a control group, mean age: 33.0 (±5.4) y/o and 31.4 (±6.7) y/o. The min. week of miscarriage in women with PL was 6., while max. was 28. In women with and without PL number of pregnancies was 1.4 (±1.1) and 1.2 (±0.8), respectively. All included pregnancies were anembryonic. Characteristics of the group of women with PL are shown in Table 1, as we previously reported in Adler et al. (2018).

*DNA extraction and genotyping*

Genomic DNA from buccal swabs was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Two SNPs: rs9468 and rs1800547, were selected from the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP). Genotypes were determined by real-time PCR using StepOneTM Real-Time PCR System, Applied Biosystems and TaqMan SNP Genotyping Assays (Life technologies, Assay ID: C_7563752_10 and C_7563692_10, respectively) (Applied Biosystems, Foster City, CA, USA). The data were analyzed with Taq Man Genotyper Software v. 1.0.1. For quality control purposes, approximately 10% of the samples were re-genotyped in a blinded fashion and the same results were obtained.

*Statistical analysis*

All the tests were performed using the R CRAN statistical software (version 3.4.2) (R Core Team, 2017). Statistical analysis was performed using t-test for two independent means. Haplotypes distribution was compared between groups. $P < 0.05$ was considered statistically significant. The distribution of genotypes was determined in women with and without PL, and comparison was made by the Kruskal-Wallis Test and Fisher's Exact Test. The function HWE.chisq for two alleles from package genetics was performed (Warnes et al., 2013).

## Results and Discussion

Two haplotypes of the 17q21.31 inversion in women from B&H with PL and control group (mothers with at last one live-born child) were identified. In women with and without pregnancy loss we identified 115 and 122 *H1H1*, 37 and 27 *H1H2* and 2 and 5 *H2H2* haplotypes (see table 2.). There were no significant differences between the distributions of haplotypes in women with and without PL (P-value = 0.25). In women with and without pregnancy loss the distribution of *H1* haplotype was 267 (86.7%) and 271 (88.0%), while the distribution of *H2* was 41 (13.3%) and 37 (12.0%). Due to the small sample size we assessed the expected value of genotype prevalence in both groups, P values were 0.611 and 0.032.

The results of our study showed that women with *H1H2* haplotype in comparison to women with *H2H2* haplotype have more children ($n_{avg.} = 1.54$ vs. $n_{avg.} = 1.29$). Nevertheless, statistically significant differences in our study group (n=308) were not found. Similar results were obtained in the Stefansson et al. (2005) study (n=29137). It is important to mention that in Stefansson et al. (2005) study both women (n=16959) and men (n=12178)

**Table 2.** Distribution of allele and genotype frequencies of the 17q21.31 inversion in women with and without PL

| | Observed frequency, n (%) | | HW- expected | | |
|---|---|---|---|---|---|
| Inversion 17q21.31 | Women with PL[1], n=154 | Women without PL[2], n=154 | Women with PL[1], n=154 | Women without PL[2], n=154 | *P value* |
| Genotypes, n (%) | | | | | |
| *H1H1* | 115 (74.7) | 122 (79.2) | 119.2 (75.2) | 119.2 77.4) | 0.61[1]; 0.03[2], respectively |
| *H1H2* | 37 (24.0) | 27 (17.5) | 36.1 (23.1) | 32.6 (21.1) | |
| *H2H2* | 2 (1.3) | 5 (3.3) | 2.7 (1.8) | 2.2 (1.4) | |

were subjects. Average number of offspring in subjects with *H1H2* haplotype in their study was 0.0796. To the best of our knowledge we were the first to analyze the distribution of *H1* and *H2* haplotypes in B&H population and its impact on increased number of offspring. We also tested possible relationship between *H1* and *H2* haplotypes of the 17q21.31 inversion and PL. Association of *H1* and *H2* haplotypes and pregnancy loss was not previously examined. In our study relationship between pregnancy loss and haplotypes of the 17q21.31 was not found. Inversions are structural variants in genome linked to phenotypic differences and adaptation of organisms through ages. They often have almost identical inverted repeats at their breakpoints which makes their detection very challenging. (Alkan et al., 2011; Puig et al., 2015). Therefore, there is still very little information on inversions in the human genome. It is unknown how many polymorphic inversions exist in humans, what are their global frequencies and distributions and what features are they related to (Martínez-Fundichely et al., 2014).

In the last decade a great effort has been devoted to characterizing the human genome (Auton et al., 2015). Unfortunately, a significant part of the genetic risk for common and complex diseases still remains unclear (Eichler et al.2010). It should also be noted that even if the studied were conducted, not all variants have been studied at the same level of detail. Based on the above mentioned the role of inversion still remains to be solved.

Major limitation of the presented study is small sample size. It is important to point out that the distribution of *H2* haplotype observed in our study is congruent to those reported in previously mentioned studies from European populations.

## Conclusion

In Bosnian population, haplotype *H2* of the 17q21.31 was not associated with number of children nor pregnancy loss as there were no significant differences in the distributions of haplotypes in women with and without PL.

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## Conflict of Interest

The authors state there is no conflict of interest.

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*Short communication*

# FINDINGS FROM ACGH IN PATIENT WITH PSYCHOMOTOR DELAY-CASE REPORT

Vanja Vidović[1*], Nela Maksimović[2], Tatjana Damnjanović[2], Biljana Jekić[2], Irina Milovac[1], Milka Grk[2], Stojko Vidović[1]

[1]*University of Banja Luka, Faculty of Medicine, Department of Human Genetics, Banja Luka, Bosnia and Herzegovina*
[2]*University of Belgrade, Faculty of Medicine, Institute of Human Genetics, Belgrade, Serbia*

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***Correspondence**
E-mail:
vanjatmc@hotmail.com
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*Array Comparative Genomic Hybridization, deletion, duplication*

## Abstract

Initial testing of children with psychomotor delays considers karyotype analysis and metabolic tests. However, introduction of Array Comparative Genomic Hybridization (ACGH) has become the standard method of diagnostics worldwide. ACGH is a highly sensitive method which enables detection of unbalanced chromosomal aberrations and aneuploidies. In this case report, a patient is a sixteen year old girl born to unrelated parents with mild mental retardation and psychomotor delay, hyperacusis, epilepsy, silent nasal speech, clinodactyly of the V finger on left hand, as well as low set ears. Patient had a karyotype interpreted as normal using GTG band analysis. Array CGH was performed using Agilent SurePrint G3 custom CGH+SNP Microarray 8x60K (UCSC, hg19, NCBI Build 37, February,2009). Results were analyzed by CytoGenomics 3.0 Agilent software. Results of aCGH revealed clinically significant duplication of 17q25.1-q25.3 region with the size of~7.96Mb. Within the duplicated region 217 genes are present, of which 36 are described as OMIM morbid. Duplications of similar size are described in DECIPHER date base in patients with psychomotor delay, hyperactivity and neoplasm of CNS. Besides duplication, a ~755kb clinically significant deletion was detected in the 17q25.3 region. Deletion involves 18 genes of which 2 are described as OMIM morbid: TBCD (MIM604649) and ZNF750 (MIM610226). Patient with similar deletion was described in DECIPHER date base with notable psychomotor delay. Based on these results FISH analysis is recommended for both parents in order to determine the possible carrier of inversion in the region of 17qter.

## Introduction

Developmental delay (DD) and intellectual ability (ID) is characterized by a decrease in cognitive and adaptive features and affects 1-3% of the world population, with often omitted accurate diagnosis (Bartnik et al., 2014; Flint & Knight, 2003). In about 65-80% of cases the cause of the disease remains unknown. The diagnosis of DD/ID usually arises if an IQ is less than 70, which means that most of these patients are diagnosed at an early age (Uwineza et al., 2014). However, in the most cases the diagnosis is based on the motor, speech, cognitive and social stalled. When DD/ID are associated with multiple congenital anomalies, the most common cause of the disease are chromosomal aberrations (Bartnik et al., 2014). The etiology of the disease is heterogeneous and it can be caused by the genetic factors, as well as by environmental factors (Grayton et al., 2012).

Besides Mendelian DD/ID, one of the most common causes of these conditions are submicroscopic chromosomal rearrangements and copy-number variants (CNVS) (Regier et al., 2010). In the last few years' application of Array Comparative Genomic Hybridization (ACGH) significantly improved clinical diagnostics in patients with DD/ID, congenital anomalies, autism spectrum disorders, and dysmorphic features (Girirajan et al., 2012). Improvement in ACGH resolution enabled detection of CNVS ranging in size from megabases to few kilobases (Rodriguez-Revenga et al., 2007). The detection rate of clinically relevant CNVS varies between 15% and 18% (Miller et al., 2010).

## Material and methods

In this case report, a patient is a sixteen year old girl born to unrelated parents with mild mental retardation and psychomotor delay, hyperacusis, epilepsy, silent nasal speech, clinodactyly of the V finger on left hand, as well as low set ears. Prior to Array CGH analysis, a cytogenetic analysis was performed using a standard method of G-banding according to the International System for Human Cytogenetic Nomenclature (ISCN 2015) (Haffer et al., 2013). ACGH was performed at the Faculty of Medicine, Institute of Human Genetics, Belgrade, Serbia. Isolation of genomic DNA was extracted from 5 mL of peripheral blood according to (Miller et al., 1988). Array CGH was performed using Agilent SurePrint G3 custom CGH+SNP Microarray 8x60K (UCSC, hg19, NCBI Build 37, February,2009), according to the manufacturer's instructions. This platform contains 60-mer oligonucleotide probes spanning the entire human genome with 41 Kb overall median probe spacing (33 Kb in Refseq genes). After hybridization results were analyzed by CytoGenomics 3.0 Agilent software.

## Results and Discussion

Patient had a karyotype interpreted as normal using GTG band analysis. Results of ACGH revealed clinically significant duplication of 17q25.1-q25.3 region with the size of~7.96Mb. Within the duplicated region 217 genes are present, of which 36 are described as OMIM morbid. Besides duplication, a ~755kb clinically significant deletion was detected in the 17q25.3 region. Also, analysis revealed a few CNVS which are described as normal variations in the Database of Genomic Variants.

In the last several years ACGH has become a first tier clinical genetic test for patients with developmental delay/intellectual disability and multiple congenital anomalies. Compared to conventional karyotyping (550 bands resolution) which is able to detect chromosomal aberrations >5-10 Mb, ACGH has increased the diagnostic yield of 15-20%. For instance, a research on 54 patients, selected according to clinical criteria, molecular and cytogenetic data 26 patients (48%) were diagnosed with structural rearrangements, while in 15 patients (28%) CNVS were proved to be clinically relevant (Iourov et al., 2012). Also, research on a sample of 318 patients with a diagnosis of mental retardation and multiple congenital anomalies described the potential pathological CNVS in 52 patients (16.4%) in size of 0.25 to 15 Mb (Gijsber et al., 2009).

In our case report ACGH revealed clinically significant duplication of 17q25.1-q25.3 region with the size of~7.96Mb. Duplications of similar size are described in DECIPHER date base in patient 254723 with psychomotor delay, hyperactivity

and neoplasm of CNS, as well as in patient 255159 with psychomotor and speech delay. Also, the research of Chong et al. 2014 reported a similar duplication of 7.10 Mb of the 17q25.1-q25.3 region in a female patient with severe developmental delay, hypotonia and failure to thrive. Besides this duplication, a clinically relevant deletion was detected. Deletion of ~755kb involves 18 genes of which 2 are described as OMIM morbid. One of them is Tubulin-specific chaperone D (TBCD; MIM604649) which is related to encephalopathy, progressive, early-onset, with brain atrophy and thin corpus callosum (Miyake et al., 2016). The second gene within this deletion is Zinc finger protein 750 (ZNF750; MIM610226) which is thought to cause Seborrhea-like dermatitis with psoriasiform elements (Birnbaum et al., 2006). Also, a patient 278987 with the deletion of this region was described in DECIPHER date base with notable psychomotor delay.

## Conclusion

This case report represents a preliminary results of our research which will include 24 patients with psychomotor delays, developmental delays, congenital anomalies and dysmorphic features which do not have a final diagnosis. The patient in this case report is a sixteen year old girl with mild mental retardation and psychomotor delay, hyperacusis, epilepsy, silent nasal speech, clinodactyly of the V finger on left hand, as well as low set ears. ACGH revealed clinically significant duplication of 17q25.1-q25.3 region with the size of~7.96Mb as well as a ~755kb clinically significant deletion in the 17q25.3 region. Also, analysis revealed a few CNVS which are described as normal variations. Characterization of the chromosomal aberrations detected by ACGH will contribute to successful diagnosis, adequate genetic counseling as well as the usage of specific therapeutic procedures. Also, this is the first study in our population, and currently there is no database of normal copy number variations which are characteristic for each population.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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*Short communication*

# GENETIC CHARACTERIZATION OF AUTOCHTHONOUS VARIETIES OF "LUBENIČARKA" PEAR USING AFLP MARKERS

Ljubomir Radoš[1], Bojana Vučković[1], Tanja Krmpot[1*], Aleš Vokurka[2], Zlatan Kovačević[1]

[1]*University of Banjaluka, Faculty of Agriculture, Banja Luka, Bosnia and Herzegovina*
[2]*University of Zagreb, Faculty of Agriculture, Zagreb, Croatia*

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***Correspondence**
E-mail: tanja.krmpot@agro.unibl.org

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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.

## 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 to 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 Mićić 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 (Mićić 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; Mićić 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 (Akcay 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 $MgCl_2$ and 50 mM KCl, with 0.2 mM of each dNTP, 0.5 U Taq-polymerase (Sigma), and 0.25 uM 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 VeritiTM 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 $MgCl_2$ 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 VeritiTM 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* ↓↓↓ |
|---|---|---|---|
| **Adapter *Eco*RI** | | 5'- ctc gta gac tgc gta cc -3' | 6Fam E32 - M47 (*p1*) |
| | | 5'- aat tgg tac gca gtc -3' | 6Fam E33 - M48 (*p2*) |
| | | | 6Fam E32 - M49 (*p3*) |
| **Primer E+1 (pre-amplification)** | E01 | 5'- gac tgc gta cca att c **a** -3' | 6Fam E32 - M59 (*p4*) |
| **Primer E+3 (selective amplification)** | 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*) |
| | | | Vic E40 - M61 (*p10*) |
| **Adapter *Mse*I** | | 5'- gac gat gag tcc tga g -3' | Vic E40 - M50 (*p11*) |
| | | 5'- tac tca gga ctc at -3' | Vic E40 - M60 (*p12*) |
| | | | Vic E40 - M62 (*p13*) |
| **Primer M+1 (pre-amplification)** | M02 | 5'- gat gag tcc tga gta a **c** -3' | Vic E40 - M59 (*p14*) |
| **Prajmer M+3 (selective amplification)** | 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 Mićić 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.

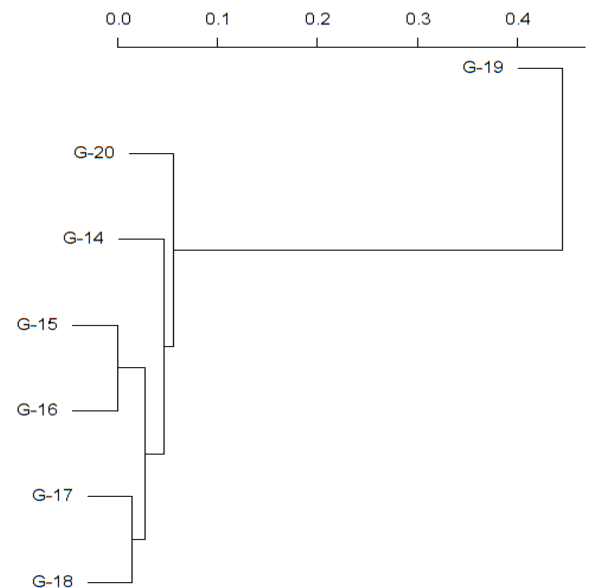


**Figure 1.** The dendrogram grouping the genotypes in accordance with the coefficients of difference

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

Banjaluka region is characterized by a very rich and varied diversity of old and autochthonous varietes of pears, which represent a very inportant genetic potencial 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 Banjaluka 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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