ISSN 2566-2937

Genetics • Genomics • Genetic engineering • Biotechnology • Bioinformatics

Genetics & Applications

Vol. 1, No. 1
June, 2017

G&A

The Official Publication of the
Institute for Genetic Engineering and Biotechnology
University of Sarajevo

ISSN 2566-2937

Genetics & Applications

Volume 1, Number 1

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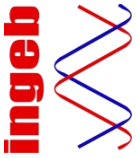

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Impressum

Cover photography

Stoneflies (Plecoptera) in river Ribnica, Kakanj. Provided by: *Semir Dorić & Adnan Čučuković*. Copyright by the University of Sarajevo, Institute for Genetic Engineering and Biotechnology, B&H

Publisher

Institute for Genetic Engineering and Biotechnology, University of Sarajevo

Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

www.ingeb.unsa.ba

Phone: +387 33 220-926

Fax: +387 33 442-891

ingeb@ingeb.unsa.ba

Contents

Editor´s Preface

Science based phase of the development of biodiversity research in Bosnia and Herzegovina (B&H) begins with the foundation of the Department of natural history within National Museum of Bosnia and Herzegovina (1988), whose tradition gave rise to all the fields of biology, biomedicine and biotechnology in our state.

The articles published in the 1930s are usually declared as pioneering works in genetics in B&H; however, the first considerable contributions to the understanding of genetic constitution of B&H population emerge in the 1960s. Although, research of similar type have been initiated at the end of XIX century, before the establishment of genetics as a modern science. Austrian military physicians have than described frequencies of highly heritable traits in conscripts from five contemporary administrative counties. They observed eye, hair and skin color, and did not delve into genetic basis of the traits which, at that time, was unknown.

Population-genetic research into qualitative variation within BH population was particularly vigorous and organized over the past four decades, when basic parameters of genetic structure were described.

The levels of data analysis are linked to particular timeline. Beginning with the onset of anthropo-genetic research in this area those include:

(1) Analysis of gene and phenotype frequencies in certain phenotype systems in human:
- *Period 1934-1935 and 1953-1966 includes the analysis of ABO, Rh and MN systems;*
- *Period 1967-1976 includes analyses of numerous biochemical, physiological and morphological traits of mainly urban population;*

(2) Complex research into inter-populational genetic distance in mainly rural populations, beginning in 1977.

The formation of the Institute for Genetic Engineering and Biotechnology of Sarajevo University and maturation of a new generation of geneticists, established in referent databases, is a turning point in the development of B&H genetics.

The period of molecular-genetic research into intra-populational and inter-populational variation of genetic markers in human, animal and plant populations in B&H includes a series of studies reflected in completion of numerous research projects.

Earlier research into genetic structure of BH human, animal and plant populations are based on the observation of nuclear and mitochondrial DNA markers using various methods.
In the observed groups of local human, animal and plant populations, allelic frequencies and indicators of nuclear genetic heterogeneity were analyzed on the basis of as many as 15 genetic markers. B&H geneticists of the first and the second generation, with dozen exceptions, have published the results of the indicated research predominantly in local journals, unavailable to the wider scientific community.

Widening of the circle of classical and molecular geneticists and the establishment of Genetic Association in B&H have necessitated an introduction of an aspiring local journal of genetic

research (in English), with competent international editorial board that would include established researchers in the various fields of genetics.

We hereby expose this first issue of Genetics & Applications (G&A) to the public scrutiny, and invite all the geneticists of the world to join us in our endeavor on advancing its quality and international reputation.

Sarajevo, May 2017

Editor in Chief:
Prof. dr. Kasim Bajrović

President:
Prof. dr Rifat Hadžiselimović

GENETIC IDENTITY OF RASPBERRY 'POLANA' PLANTLINGS EXAMINED USING MICROSATELLITE MARKERS

Fuad Gaši[1*], Adnan Hodžić[1], Mirza Hadžiavdić[1], Mirsad Kurtović[1], Jasmin Grahić[1], Lejla Lasić[2], Belma Kalamujić Stroil[2], Naris Pojskić[2]

[1]*University of Sarajevo, Faculty of Agriculture and Food Sciences, Sarajevo, Bosnia and Herzegovina*
[2]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*

***Correspondence**
E-mail: fudo01@yahoo.com

Received
January, 2017
Accepted
April, 2017
Published
June, 2017

Short communication

Abstract

Raspberry cultivars are clonally propagated and therefore all plants belonging to a single cultivar represent the same genotype. Cultivar integrity of raspberry plantlings placed on the market in Bosnia and Herzegovina (B&H) is based on examining of morphological traits, which is not a reliable tool for genetic identification. In this study plantlings declared as cultivar 'Polana' were genotyped using seven microsatellites, in order to gain preliminary insight into the genetic integrity of raspberry plantlings marketed in B&H. Plant tissue (leaves) from 10 raspberry plants were randomly sampled from a batch of plantlings sold by major fruit nursery in Bosnia and Herzegovina. Along with these samples, four reference cultivars with confirmed identity ('Polka', 'Autumn Bliss', 'Heritage' and 'Polana') were also included in the study. Seven primer pairs amplified 31 alleles, or on average 4.4 alleles per locus. UPGMA cluster analysis, based on the Jaccard similarity coefficient, revealed that among the ten samples declared as 'Polana' plantlings only five were genetically identical to any of the other samples. The cluster analyses also exposed that none of the ten samples declared as 'Polana' seedlings were in fact identical or even closely related to the 'Polana' reference cultivar or any of the other reference cultivars. These findings clearly show that the genetic identity of primocane raspberry plantlings , currently sold in Bosnia and Herzegovina, needs to be tested using objective and reliable methods rather than simple morphologic observation.

Key words: *cultivar integrity, morphological traits, DNA*

Introduction

Red raspberry (*Rubus idaeus*), along with strawberry, represents the most commercially important berry fruit currently cultivated in Bosnia and Herzegovina (B&H). Raspberry cultivars are clonally propagated and therefore all plants belonging to a single cultivar possess an identical genetic makeup (same genotype). In order for raspberry plantlings to be placed on the market in B&H, they must be supervised during the nursery production in order to ensure the cultivar integrity. However, this supervision is based on examining morphological traits,

which are oftentimes not a reliable tool for the verification of genetic identity. Unlike morphological traits, microsatellite DNA markers have proven to be an excellent tool for examining the cultivar integrity among raspberry genotypes (Fernandez-Fernandez et al., 2011). A number of different microsatellites or SSRs (Simple Sequence Repeats) have been developed for R. idaeus (Graham et al., 2002; Castillo et al., 2010) and are available for this purpose. Microsatellite markers have previously been used in genetic studies of several fruit crops in Bosnia: apple (Gasi et al., 2010; Gasi et al., 2013a, Gasi et al., 2013d), pear (Gasi et al., 2013b), plum (Halapija-Kazija et al., 2014) and cherry (Gasi et al., 2013c). However, until now SSRs have not been used for genetic analyses of berry fruits in this country.

In this study, genetic identity of plantlings declared as cultivar 'Polana' were genotyped using microsatellites, in order to gain preliminary insight into the genetic integrity of raspberry plantlings marketed in Bosnia and Herzegovina.

Table 1. Seven SSR markers developed by Castillo et al. (2010), used in this study

SSR marker	forward primer	reverse primer
RhM001	GGTTCGGATAGTTAATCCTCCC	CCAACTGTTGTAAATGCAGGAA
RhM003	CCATCTCCAATTCAGTTCTTCC	AGCAGAATCGGTTCTTACAAGC
RiM019	ATTCAAGAGCTTAACTGTGGGC	CAATATGCCATCCACAGAGAAA
RhM023	CGACAACGACAATTCTCACATT	GTTATCAAGCGATCCTGCAGTT
RhM011	AAAGACAAGGCGTCCACAAC	GGTTATGCTTTGATTAGGCTGG
RhM043	GGACACGGTTCTAACTATGGCT	ATTGTCGCTCCAACGAAGATT
RiM015	CGACACCGATCAGAGCTAATTC	ATAGTTGCATTGGCAGGCTTAT

Materials and methods

Plant tissue (leaves) from 10 raspberry plants, randomly sampled from a batch of plantlings sold by a major fruit nursery in Bosnia and Herzegovina, were used for the isolation of genomic DNA applying CTAB method (Doyle and Doyle, 1987). All of the sampled plants were declared by the nursery to be the plantlings derived from the primocane raspberry cultivar 'Polana'. Along with these samples, four reference cultivars with confirmed identity ('Polka', 'Autumn Bliss', 'Heritage' and 'Polana') were also included in the study. Seven primer pairs (Table 1) used for SSR amplifications have previously been published by Castillo et al. (2010). Polymerase chain reaction (PCR) amplification of SSR sequences was performed in a Veriti TM Thermal Cycler (Applied Biosystems, Foster City, CA) using fluorescently labelled primers. All PCR amplifications were performed as described in Castillo et al. (2010). The detection of SSR products was conducted on ABI 310 automated sequencer (Applied Biosystems). PCR product (1 µL) was added to a master mix containing 9 µL of deionized formamide and 0.5 µL GeneScan-350 Rox size-standard (Applied Biosystems). Samples were heated at 95 ˚C for 5 min and immediately cooled down on ice. SSR profiles were scored using GeneMapper Software ID v3.2 (Applied Biosystems). UPGMA cluster analysis, based on Jaccard similarity coefficient, was calculated using the obtained SSR data, in NTSYS software (Rohlf, 1993) and visualized by MEGA 5 software (Molecular Evolutionary Genetics Analysis), (Tamura et al., 2011).

Results and discussion

Seven primer pairs amplified 31 alleles (Table 2), or on average 4.4 alleles per locus, among all the 14 analysed raspberry samples. Higher values for the average alleles per locus (7.9), among the same SSR loci, have been published by Castillo et al. (2010). However, in that study 48 different raspberry genotypes were examined. Overall, the seven primer pairs used in our research provided sufficient variation for clear distinction among four reference cultivars. The same cluster analyses revealed that among the ten samples declared as 'Polana' plantlings, only five were genetically identical to any of the other samples. Considering that raspberry plantlings are clonally propagated, all true offspring should share the same SSR profile. The cluster analyses also revealed that none of the ten samples declared as 'Polana' plantlings were in fact identical or even closely related to 'Polana' reference cultivar or any of the other

Table 2. SSR profiles (allele sizes expressed in base pairs) of 10 raspberry plants, declared as plantlings of the cultivar 'Polana', and four reference cultivars, obtained through the analyses of seven SSR loci.

Genotypes	**RhM001**		**RhM003**		**RiM019**		**RhM023**		**RhM011**		**RhM043**		**RiM015**	
'Polka'	239	241	202	202	180	182	195	195	289	291	371	373	351	351
'Autumn Bliss'	227	229	191	193	182	217	187	195	279	279	373	373	349	351
'Heritage'	227	236	193	202	180	182	187	195	281	291	371	371	349	351
'Polana' ref.	236	236	200	202	182	195	187	195	281	281	371	373	351	351
Sample 1	227	236	200	202	174	182	187	195	279	279	373	373	349	351
Sample 2	227	236	195	202	170	178	187	195	285	285	373	373	349	359
Sample 3	227	236	200	202	174	182	187	195	279	279	373	373	349	351
Sample 4	227	236	200	202	174	182	187	195	279	279	373	373	349	349
Sample 5	227	236	200	202	182	182	187	195	279	279	373	373	349	351
Sample 6	227	236	200	204	180	182	187	195	285	285	373	373	349	349
Sample 7	227	236	191	193	174	182	187	195	279	279	373	373	349	351
Sample 8	227	236	191	193	174	182	187	195	279	279	373	373	349	351
Sample 9	227	236	191	193	174	182	187	195	279	279	373	373	349	351
Sample 10	227	236	200	202	182	182	187	195	279	279	373	373	335	339

UPGMA cluster analysis, based on Jaccard similarity coefficient, revealed that among the reference cultivars, 'Heritage' and the reference 'Polana' samples clustered the closest (Figure 1). Similar findings have already been reported by Bassil et al. (2012). Also, considering that 'Heritage' is a parent of the cultivar 'Polana' this result was entirely expected.

reference cultivars. The obtained results indicate that investigated samples are at least in partgenerative offspring, which probably arose through spontaneous hybridization between unknown raspberry genotypes. These findings clearly show that the supervision of nursery production, based on morphological traits, does not guaranty cultivar integrity.

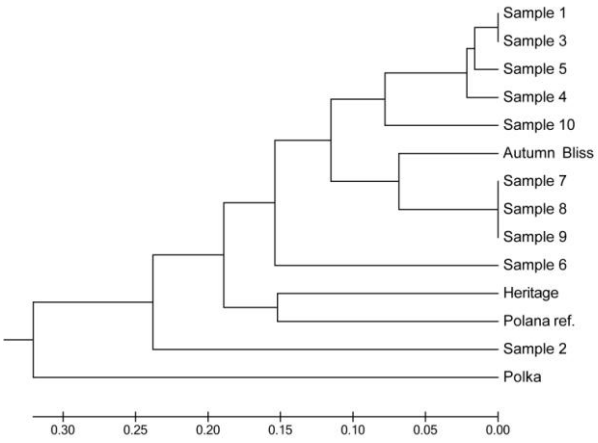

Figure 1. UPGMA cluster analyses of 10 raspberry plants, declared as plantlings of the cultivar 'Polana', and four reference cultivars

Conclusions

The genetic identity of primocane raspberry seedlings, currently sold in Bosnia and Herzegovina, needs to be tested using a more objective and reliable method than simple morphologic observation. The results of this study indicate that DNA markers are a necessary tool for this task, among which microsatellites can be considered as highly suitable.

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AN OPTIMIZED DNA ISOLATION PROTOCOL ENABLES AN INSIGHT INTO MOLECULAR GENETIC BACKGROUND OF ENDEMIC *Moltkia petraea* (Tratt.) Griseb. FROM BOSNIA AND HERZEGOVINA

Jasna Hanjalić [1*], Semir Dorić[1], Lejla Lasić[1], Belma Kalamujić Stroil[1], Selma Hasičić[1], Naris Pojskić[1]

[1]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*

***Correspondence**
E-mail: jasna.hanjalic@ingeb.unsa.ba

Received
January, 2017
Accepted
May, 2017
Published
June, 2017

Research article

Abstract

The Dinaric endemic plant species *Moltkia petraea* (Tratt.) Griseb. is often called a "living fossil" of ancient Tertiary flora, with great importance for Bosnia and Herzegovina's biodiversity. Considering its narrow and limited distribution range, insufficient data on the molecular background of this species is given so far. Due to the presence of various secondary metabolites that interfere with the DNA, isolation of nucleic acids from plant cells is known to be challenging. Even in closely related species it is necessary to optimize DNA isolation protocol in order to obtain high quality PCR amplifiable DNA. We collected 91 samples from five populations in Herzegovina. Doyle and Doyle (1987) CTAB protocol was modified by adding vitamin C (ascorbic acid) to the cell lysis buffer to improve DNA yield and quality. *trn*L(UAA) intron and *nr*DNA (*ITS1*, *ITS2*) molecular markers were applied to demonstrate amplifiability of isolated DNA and elucidate the intra- and interpopulation genetic diversity. Our results suggest a successful PCR amplification for 81% of the analyzed samples. PCR-RFLP analysis of *trn*L(UAA) revealed that all individuals in five populations have the same haplotype based on the obtained enzymatic profile for three enzymes (*Taq*I, *Hinf*I, *Hind*II). Alignment and comparison of ITS sequences didn't reveal any hypervariable portion that could be informative in elucidating the genetic diversity of *M. petraea* populations. Further studies with additional application of microsatellite loci, RAPD and AFLP methods are necessary in an attempt to get insights into the genetic diversity of *M. petraea*.

Key words: *Moltkia petraea, trnL, nrDNA, DNA isolation*

Introduction

The angiosperm family Boraginaceae includes approximately 1600 species distributed among some 110 genera, characterized by a scorpioid cymose inflorescence (Buys and Hilger, 2003). Genus *Moltkia* Lehm. comprises five species (Cohen, 2014), with *Moltkia petraea* (Tratt.) Griseb growing mostly in Mediterranean and Submediterranean regions (Kremer et al., 2016). This species is a dense, dwarf shrub, up to 40 cm high, with deep violet–blue tubular flowers, in simple forked or branching revolute cymes,

blooming from spring well into the summer (Fernandes, 1972; Polunin, 1987). It thrives on calcareous cliffs, in rock crevices, with altitude distribution ranging from near the sea level to 1500 m a.s.l., although it has been recorded at 2000 m a.s.l. on Mt. Durmitor in Montenegro (Trinajstić, 1974). While *M. petraea* withstands wide range of ecological parameters, it finds the optimal conditions at altitudes between 300 and 900 m a.s.l. (Marković, 1994). It is probably an ancient or even relict species (Turrill, 1929). The current presence of *M. petraea* in gorges is probably due to its preservation within these protected areas during the last Ice Age (Georgiou et al., 1999). This species is considered an element of the endemic Ilyric-Balkan flora restricted to Bosnia and Herzegovina, Albania, Montenegro, Croatia, Macedonia and Greece (Jerković et al., 2017). In B&H, it is present in the mountain regions: Prenj, Čvrsnica, Plasa, Čabulja, Velež, Žaba, Bijela Gora and Orijen (Šoljan et al., 2009).

Internal transcribed spacer (ITS) is commonly used as one of the molecular markers in phylogenetical studies in Boraginaceae (Cecchi and Selvi, 2009; Cohen, 2014; Chacón et al., 2016), an unplaced group in the current angiosperm phylogeny. The knowledge on intraindividual polymorphism of ITS in Boraginaceae is scarce, although there is evidence of limited intraindividual polymorphism (Kook et al., 2014). Cecchi and Selvi (2009) reported a phylogenetic study based on ITS molecular markers. According to them, genus *Moltkia* is a monophyletic and somewhat isolated group belonging to single clade with no direct relationship to the genera of other clades. The characteristic deletion in *ITS1* (11-bp deletion, pos. 235–245) shared by the five species of this genus is a strong argument for their common ancestry.

The only effort at surveying population (genetic) diversity of *M. petraea* has been made by Šamec (2013), who used RADP markers to analyze *M. petraea* from Croatia. However, the author reported that any attempt at getting an amplicon failed. So far, *M. petraea* from Bosnia and Herzegovina has only been analyzed for the influence of soil properties on phenolic compound accumulation (Kremer et al., 2016) but no data on its genetic diversity exist.

Critical step for molecular characterization of any plant species is DNA isolation. Generally, it is quite difficult to extract and purify high-quality DNA because of high content of secondary metabolites (tannins, alkaloids, and polyphenols), polysaccharides and proteins in plants that mostly act as DNA polymerase inhibitors. These compounds precipitate along with DNA, degrade its quality, reduce yield and often render the sample non-amplifiable and DNA unstable for longer period (Sarwat et al., 2006, Hassan et al., 2012). As these compounds are ubiquitous in plant DNA extracts, numerous isolation protocols have been modified and used in various combinations. Due to the chemical heterogeneity among species, even closely related species may require different isolation protocols (Sharma et al., 2002).

The objectives of this study were (i) to obtain high quality DNA from fresh leaves of M. petraea with rapid, cost efficient and simple method for its extraction and purification, and (ii) to inspect the population (genetic) diversity using the trnL and ITS molecular genetic markers.

Materials and methods

Sample Collection

The total of 91 samples were collected from five regions in Herzegovina (Table 1, Figure 1) and stored at -20 ˚C. For the purposes of DNA analysis, sampling procedure included: (i) taking into account the distance between individuals; (ii) sampling the branches (of each

individual) with a few leaves on it respecting the fact that it is a rare plant species; (iii) packing plant material into paper bags (each specimen separately to avoid cross-contamination).

Table 1. Absolute and relative number of individuals collected by region and altitude

Region	Number of individuals	Elevation (m a.s.l.)	(%)
Mostarska Bijela	8	600	9
Grabovica	31	400	34
Most Begića i Begovića	20	200	22
Tunel	19	200	21
Drežnica	13	200	14

DNA extraction and qualitative-quantitative analysis

Frozen and fresh plant tissue (15 mg per sample on average) was grounded using mortar and pestle. Different protocols for DNA extraction were used: CTAB protocol (Doyle and Doyle, 1987); DNeasy Plant Mini Kit (Qiagen), protocol described by Jobes et al. (1995) and eventually the optimized procedure based on CTAB. The quality of genomic DNA was analyzed using agarose gel electrophoresis. Five microliters of genomic DNA were run in 1.5 % (w/v) agarose gel, in 1x SB (Sodium borate) buffer, pH8 (Brody and Kern, 2005) and visualized under UV light after staining with Midori green (*Nippon Genetics Europe*). Spectrophotometry was used to determine the concentration and quality of isolated DNA (Gallagher, 1994).

Molecular markers

Chloroplast trnL and *nr*DNA regions were used to demonstrate amplifiability of isolated DNA and afterwards observed for the analysis of genetic diversity. Amplification of *trn*L (UAA) intron was performed in an optimized PCR reaction using primers described by Taberlet et al. (1991) in 15 µl reactions consisting of 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM MgCl2 and 1 unit of TaqGold DNA polymerase (*Thermo Fisher Scientific*). PCR amplification was carried out in 30 cycles (5 min at 95°C, 45s denaturation at 95°C, 30s annealing at 51°C, 45s extension at 72°C and 10 min final extension at 72°C). Successfully amplified *trn*L (UAA) intron was digested with restriction enzymes (*Taq* I, *Hpy*F3 I, *Hinf* I, *Hind* III, *Hind* II, *Rsa* I, *Ecor* I, *Ava* II, *Ban* I and *Alu* I) in order to create RFLP profiles. Digestion was performed in individual reactions according to the manufacturers' instructions (BioLabs New England).

Figure 1. Geographical location of sampling sites

*nr*DNA regions of *ITS1* and *ITS2* (with accompanying 5.8S rRNA) were amplified in separate reactions using primers previously described by White et al. (1990). Amplifications were performed in 35 µl reactions consisting of 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM MgCl2 and 1 unit of TaqGold DNA polymerase (*Thermo Fisher Scientific*). PCR parameters were 3 min at 95°C, 30s denaturation at 95°C, 30s annealing at 50°C, 1

min extension at 72°C and 10 min final extension at the same temperature with the total of 30 cycles. PCR products were sequenced by Macrogen Inc. Europe as a part of their regular capillary DNA sequencing services.

Sequence and genetic data analysis

Sequence identification analysis for *nr*DNA from *M. petraea* was performed using the FASTA program (Pearson, 1994). BLAST network service (Benson et al., 2003) in GenBank at NCBI was used for final sequence identification, searching for the best identity and similarity scores in local databases. Sequencing reads were assembled using DNASTAR's Lasergene software EditSeq (Burland, 2000). Electropherograms were examined manually for sequencing errors. Multiple sequence alignment analyses for *nr*DNA sequences were performed using ClustalW Ver.1.6 (Thompson et al., 2011) under default parameters. MSA analyzed sequences and outputs were optimized using Jalview 2.9.0b2 (Waterhouse et al., 2009) and edited by Bioedit v5.09 (Hall, 1999).

Analysis of genetic data included the generation of haplotypes based on PCR-RFLP (site polymorphism). ITS sequence variation within and among observed population, intra- and interpopulation nucleotide diversity, and nucleotide differentiation were estimated using MEGA6 (Nei, 1987; Nei and Kumar, 2000).

Results and discussion

Isolation of nucleic acids from plant cells is known to be challenging due to the presence of various inhibiting substances that bind and/or co-precipitate with them, interfere with the DNA isolation procedure and downstream applications such as PCR-based methods (Salzman et al., 1999). Thus, it is very difficult to create a universal isolation procedure and in most cases optimization is necessary. The content of biologically active compounds in plants depends on various factors: influence of soil traits, plant age, harvesting time, tissue type etc. (Končić et al., 2010, Kremer et al., 2016, Kolodziej and Sugier, 2013). Kremer et al. (2016) analyzed biochemical properties of *M. petraea* and reported the highest content of polyphenols, flavonoids and phenolic acids in leaves, the highest content of tannins highest in flowers, with the lowest content of the analyzed bioactive compounds in stems.

Šamec (2013) aimed to perform phytochemical and genetic analyses in four endemic species, i.e. *Teucrium arduini*, *Moltkia petraea*, *Micromeria croatica* and *Rhamnus intermedia*. Even after extensive optimization of DNA isolation protocols and application of different approaches, the author was not able to obtain an amplicon for any of the selected markers. UV and CD specter of DNA samples from M. petraea revealed the presence of unidentified compound with the maximum absorbance at 260 nm, overlapping the DNA specter.

In this study we applied several isolation protocols to obtain high quality DNA from leaves of *M. petraea* (Figure 2). Extraction using CTAB method reported by Doyle and Doyle (1987) gave low yield of DNA (Figure 2A), but samples were non-amplifiable. Application of the DNeasy Plant Mini Kit (Qiagen) (Figure 2B) and isolation protocol described by Jobes et al. (1995) (Figure 2C) resulted in DNA of even poorer quality. In order to improve DNA yield and quality, Doyle and Doyle (1987) CTAB protocol was modified. Optimization included adding vitamin C (ascorbic acid) to the cell lysis buffer (0.2%). Vitamin C is known to prevent selective binding of proteins and to have antioxidant properties. This method solved the problem of low DNA yield and co-precipitation of secondary metabolites (Figure 2D) which was proven by a successful PCR amplification for 81% of analyzed samples.

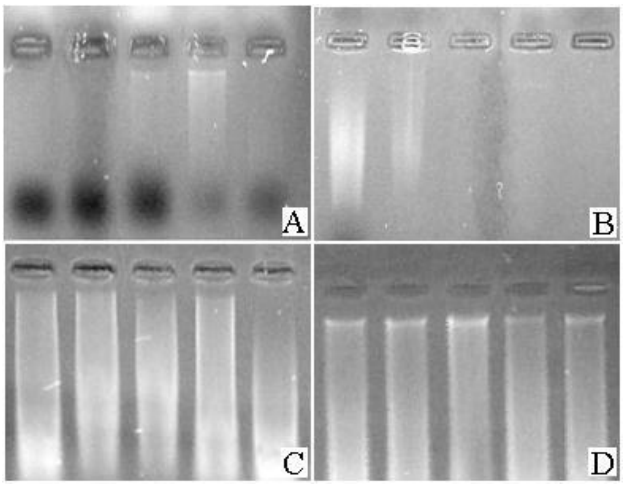

Figure 2. Electrophoretic pattern of DNA isolated by (A) CTAB protocol (Doyle and Doyle, 1987), (B) DNeasy Plant Mini Kit (Qiagen) and (C) isolation protocol described by Jobes et al. (1995). (D) Optimization of CTAB method resulted in DNA that was suitable for PCR-based analysis. The electrophoresis was performed in 1.5% (w/v) agarose gel

PCR-RFLP analysis

Chloroplast *trn*L (UAA) intron was successfully amplified for 74 out of 91 collected samples. Digestion was performed with ten different enzymes but only three of them (*Taq*I, *Hinf*I, *Hind*II) found recognition site within the amplicon. RFLP profiles obtained after digestion of *trn*L region with restriction enzymes are shown in Table 2 and Figure 3. Analysis of the restriction fragments revealed that all individuals in five observed populations have the same RFLP profile for every applied enzyme. No polymorphism (intra- or interpopulation) was detected. Consequently, haplotype distance and intrapopulation haplotype diversity equals 0.

Table 2. Restriction fragments sizes (bp) generated by digestion of the *trn*L region

***Taq*I**	***Hinf*I**	***Hind*II**
225	181	325
165	130	203
130	105	-

The *trn*L (UAA) intron is not the most variable non-coding chloroplast region due to its catalytic function and secondary structure formations, but studies based on RFLP polymorphisms of this region have been reported in different plants (Taberlet et al., 2007; Ridgway et al., 2003; Spaniolas et al., 2010). The advantage of this molecular marker is easy cross-amplification in a large number of plants due to highly conserved primers, i.e. flanking regions. However, Taberlet et al. (2007) reported its relatively low resolution (67.3%) at the species level in plants, and it indeed proved to be insufficiently sensitive for the detection of genetic diversity within species.

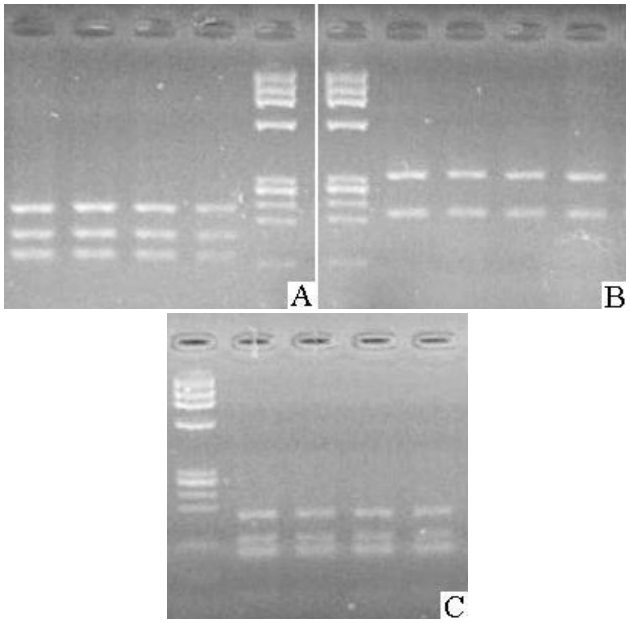

Figure 3. Restriction fragments generated using restriction enzymes A) *Taq*I, B) *Hind*II, C) *Hinf*I. The sizes of obtained fragments (given in Table 2) were determined according to ΦX174 DNA/BsuRI Ladder

Analysis of ITS sequences

The sequence nucleotide composition was successfully determined for 14 of 15 sequenced samples. Length of the *nr*DNA region (642 bp) concurs with the reference sequence in GenBank (accession number: FJ763194). One concatenated *nr*DNA consensus sequence [*ITS1*

(260 bp) - 5.8S RNA (163 bp) - *ITS2* (219 bp)] from this study was obtained and deposited in the GenBank database (accession number: KX343047).

Despite some previous evidence of ITS intrapopulation diversity in some Boraginaceae species (Kook et al., 2014), alignment and comparison of sequences (*ITS1* and *ITS2*) obtained in this study revealed the absence of polymorphisms and substitutions, as well as lack of indel mutations.

Applied nrDNA population genetic analysis showed no intrapopulation nucleotide diversity among sequences. In relation to that, the nucleotide differentiation (Nst) equals 0, suggesting that there is no genetic differentiation between the populations based on ITS sequence polymorphisms. Notably, the absence of genetic variation within and among the populations is observed, regardless of the type of marker observed.

M. petraea is considered to be paleoendemic species which, according to classical Stebbins' view (1942) are defined as depleted species, with formerly wide distribution and high genetic diversity, that had lost many or most of their biotypes, resulting in endemism (Kay et al., 2010). More often than not, restriction of habitat has negative impact on genetic diversity, causing the loss of infraspecific genetic variability. This is true for number of angiosperms (Gitzendanner and Soltis, 2000; Hamrick and Godt, 1996) such as Halacsya sendtneri (Boiss.) Dörfl., another endemic member of Boraginaceae family with distribution pattern similar to that of *M. petraea*.

Study by Coppi et al. (2014) revealed relatively low total genetic diversity (HT=0.142) in this species, based on AFLP analysis, even when compared with several case-studies on rare and/or endemic angiosperms. Although ITS region proved to be very useful in elucidating phylogenetic relationships in Boraginaceae (Cecchi and Selvi, 2009; Cohen, 2014; Chacón et al., 2016), as well as in distinguishing five species of genus *Moltkia* from other Mediterranean Lithospermeae by a unique 11-bp deletion in *ITS1* (positions 235–245) (Cecchi and Selvi, 2009), it did not reveal any hypervariable portion that could be informative in elucidating the genetic diversity of populations of *M. petraea* from the locations in Bosnia and Herzegovina. Therefore, in order to get an objective insight of *M. petraea* genetic variability and to evaluate its status as a paleoendem, future studies on this species should be based on carefully selected molecular markers.

Although application of microsatellite loci as molecular markers are inevitable in plant genetic studies for diversity estimation (Vieira et al., 2016), so far no publications about detected and described microsatellite regions within the genome of *M. petraea* have been published. In such cases, it could be more suitable to apply AFLP method which is one of the most valuable tools for investigating genetic variation in plant populations because it is simple, relatively cheap, reproducible, and demands small amount of DNA which is especially convenient for the research of rare, endemic plant species. This method has been successfully applied to diploid and polyploid species from Boraginaceae family with fragmented and/or restricted distribution (Stehlik, 2003; Mengoni et al., 2006; Coppi et al., 2008).

Optionally, RAPD markers could be used, although Šamec (2013) failed to get a successful amplification in *M. petraea*, most probably because no DNA isolation protocol used in that study yielded DNA of satisfactory purity. Therefore, previous surveys on this species as well as our study showed that it is of vital importance to choose adequate approach for the

isolation of DNA since it greatly affects the success of further analytical steps.

Conclusions

Addition of vitamin C (ascorbic acid) to the cell lysis buffer solved the problem of low DNA yield, co-precipitation of secondary metabolites and provided high quality DNA useful for further molecular genetic analysis of *M. petraea*. However, no genetic diversity within and among populations of *M. petraea* in Bosnia and Herzegovina was found in this research, based on the applied markers. This may be due to the combination of these factors: (i) observed molecular genetic markers are not sensitive enough to detect intra- and interpopulation genetic variation of *M. petraea*; (ii) observed populations represent part of an old (relict) population which has highly conserved *trn*L and ITS regions. Therefore, we suggest that further investigations of this species should employ more methods for evaluation of genetic variability in endemic plants such as AFLP or RADP fingerprinting.

Acknowledgment

This research was funded by the Environmental Fund of the Federation of Bosnia and Herzegovina, grant no. 01-02-1069/2012.

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MICROSATELLITE DIVERSITY OF CROSSBRED HORSES RAISED IN BOSNIA AND HERZEGOVINA

Dunja Rukavina[1*], Danica Hasanbašić[1], Belma Kalamujić Stroil[2], Naris Pojskić[2]

[1]*University of Sarajevo, Veterinary Faculty, Sarajevo, Bosnia and Herzegovina*
[2]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*

***Correspondence**
E-mail: dunja.rukavina@vfs.unsa.ba

Received
February, 2017
Accepted
May, 2017
Published
June, 2017

Research article

Abstract

The focus of this study was microsatellite diversity of crossbred horses raised in Bosnia and Herzegovina. Genomic DNA was extracted from blood samples of 20 individuals (KBA group – 7 individuals, crosses between Bosnian and Herzegovinian mountain horse and Arabian horse; KBR group – 9 individuals, crosses between Bosnian and Herzegovinian mountain and Belgian horses, crosses between Bosnian and Herzegovinian mountain horses and Holstein, crosses between Bosnian and Herzegovinian mountain and Lipizzaner horses and KBN group – 4 individuals, crosses between Bosnian and Herzegovinian mountain horse with an unknown origin of the other parent). The samples were profiled using 17 microsatellite markers. This method consisted of multiplex PCR procedure and generated reasonable amplification across all the loci. All samples were genotyped successfully. Considering all the observed parameters, VHL20 locus showed the highest microsatellite diversity. Locus HMS7 was the least variable in KBR group, while HMS1 locus was the least diverse in KBN group. The highest microsatellite diversity in KBA group was found at AHT5 locus while HTG6 locus was the least diverse. Obtained results suggest that the investigated populations of crossbred horses from Bosnia and Herzegovina are not affected by substantial loss of genetic diversity, as indicated by the presence of reasonably high level of genetic variation. An increase in the inbreeding coefficient and sufficient heterogenity in KBN group indicate occurrence of consanguineous mating. The present research contributes to the knowledge of current status of genetic structure of the investigated crossbred horses.

Key words: *crossbred horse, genetic diversity, molecular markers*

Introduction

Microsatellites (Short Tandem Repeats - STR) are a class of genetic markers, currently the most commonly used for diversity studies in livestock (Fornal et al., 2013; Semik and Zabek, 2013). Due to their high level of polymorphism, dispersion throughout eucariotic nuclear genome and Mendelian co-dominant inheritance, microsatellites are relatively easy to score and are considered the markers of choice in equine parentage testing and individual identification (Zabek and Fornal, 2009; Moshkelani et al., 2011). Microsatellites are

regions of repeated 2 to 7 nucleotide long units that occur primarily in non-coding regions of DNA (Moshkelani et al., 2011). Microsatellites have been employed to construct linkage maps, to examine population genetic structure and genetic variation, to explore molecular evolution, in studies of gene flow, in resolution of forensic cases and as parentage testing markers (Tozaki et al., 2003; Moshkelani et al., 2011). Microsatellites loci constitute an informative source concerning population history, structure and genetic diversity and microsatellite polymorphism still plays an important role in the assessment of genetic diversity of livestock (Semik and Zabek, 2013).

In this paper we report the results of the first analysis of microsatellite diversity of crossbred horses from Bosnia and Herzegovina (B&H) using 17 microsatellite markers currently recommended by International Society for Animal Genetics (ISAG) (ISAG, 2014). Number of different alleles, observed and expected heterozygosity, polymorphic information contents, inbreeding coefficient and deviation from Hardy-Weinberg equilibrium were estimated.

Materials and methods

The study was performed on 20 blood samples of horse crossbreeds (KBA group – 7 individuals, crosses between Bosnian and Herzegovinian mountain horse and Arabian horse; KBR group – 9 individuals, crosses between Bosnian and Herzegovinian mountain and Belgian horses, crosses between Bosnian and Herzegovinian mountain horses and Holstein, crosses between Bosnian and Herzegovinian mountain and Lipizzaner horses and KBN group – 4 individuals, crosses between Bosnian and Herzegovinian mountain horse with an unknown origin of the other parent). All horses were raised in Bosnia and Herzegovina (Rukavina et al., 2015b). Blood samples were collected from *v. jugularis* using sterile venipuncture needles and EDTA vacuum containers. Genomic DNA was isolated using salting-out method that was originally developed for the isolation of DNA from human blood (Miller et al., 1988). Necessary modifications to the protocol were made in order to accommodate for different properties of horse blood as well as our laboratory conditions (3ml of blood; 10ml of Lysis buffer; 4ml of PBS; 4ml of Kern-lysis buffer; 150µl of 20% SDS; 100µl of protease and 0,5ml 6M NaCl). In total, 20 animals were genotyped for 17 microsatellite loci. This method consists of multiplex PCR procedure and shows satisfactory amplification of all analyzed fragments. Fragment separation and allele sizing were performed using ABI Prism 310 Genetic Analyzer. All the genotypes were successfully generated. Sizing of the amplified fragments was performed using GeneMapper ID v3.2 software. Number of different alleles (AN), polymorphic information content (PIC) (Botstein et al., 1980), observed heterozygosity (Ho), expected heterozygosity (HE) (Nei, 1987), inbreeding coefficient (F) (Weir, 1996) and deviation from *Hardy-Weinberg* equilibrium (HWE) (Guo and Thompson, 1992) was calculated using POWERMARKER 3.25 (Liu and Muse, 2005).

Results and discussion

The research described in this paper was the first analysis of microsatellite diversity of crossbred horses raised in B&H. All the equine microsatellite markers, reported in the study, were amplified successfully. Results for number of alleles (AN), observed heterozygosity (HO), expected heterozygosity (HE), polymorphic information content (PIC), inbreeding coefficient (F) and deviation from Hardy-Weinberg equilibrium (HWE) are given in Tables 1, 2 and 3. In KBR group the mean

Table 1. Number of alleles (AN), expected heterozygosity (HE), observed heterozygosity (HO), polymorphic information content (PIC), inbreeding coefficient (F) and deviation from Hardy-Weinberg equilibrium (HWE) at 17 microsatellite loci in KBR group

Marker	A_N	H_E	H_O	PIC	F	HWE
VHL20	7.0000	0.8333	1.0000	0.8119	-0.1111	1.0000
HTG4	5.0000	0.7222	0.6667	0.6800	0.1667	0.3010
AHT4	6.0000	0.7778	0.6667	0.7456	0.2308	0.4260
HMS7	3.0000	0.4861	0.3333	0.4235	0.3939	0.5050
HTG6	4.0000	0.5139	0.6667	0.4760	-0.2121	1.0000
AHT5	7.0000	0.7639	1.0000	0.7393	-0.2245	0.4350
HMS6	4.0000	0.5833	0.6667	0.5295	-0.0526	0.2290
ASB23	5.0000	0.7222	0.6667	0.6800	0.1667	0.3010
ASB2	4.0000	0.6528	0.6667	0.5994	0.0698	0.1430
HTG10	4.0000	0.7083	0.8333	0.6589	-0.0870	0.4800
HTG7	5.0000	0.7222	0.8333	0.6800	-0.0638	0.8100
HMS3	6.0000	0.7500	0.6667	0.7193	0.2000	0.2040
HMS2	5.0000	0.7361	0.8333	0.6920	-0.0417	0.7760
ASB17	7.0000	0.7639	0.8333	0.7393	0.0000	0.8910
LEX3	5.0000	0.7222	0.3333	0.6800	0.6000	0.0180
HMS1	3.0000	0.4861	0.6667	0.4235	-0.2903	1.0000
CA425	4.0000	0.6944	0.8333	0.6391	-0.1111	1.0000
Mean	4.9412	0.6846	0.7157	0.6422	0.0458	

Table 2. Number of alleles (AN), expected heterozygosity (HE), observed heterozygosity (HO), polymorphic information content (PIC), inbreeding coefficient (F) and deviation from Hardy-Weinberg equilibrium (HWE) at 17 microsatellite loci in KBA group

Marker	A_N	H_E	H_O	PIC	F	HWE
VHL20	6.0000	0.7551	0.8571	0.7186	-0.0588	0.7000
HTG4	6.0000	0.7653	1.0000	0.7308	-0.2353	1.0000
AHT4	5.0000	0.6735	0.8571	0.6319	-0.2000	0.5980
HMS7	4.0000	0.6633	0.7143	0.6003	0.0000	1.0000
HTG6	3.0000	0.4388	0.5714	0.3862	-0.2308	1.0000
AHT5	7.0000	0.8469	1.0000	0.8277	-0.1053	0.1720
HMS6	5.0000	0.7653	0.8571	0.7299	-0.0435	0.6170
ASB23	6.0000	0.6939	0.2857	0.6636	0.6364	0.0020
ASB2	7.0000	0.7959	0.7143	0.7719	0.1781	0.4420
HTG10	5.0000	0.7500	0.8333	0.7078	-0.0204	0.7110
HTG7	5.0000	0.7347	0.7143	0.6894	0.1045	0.1770
HMS3	6.0000	0.8163	0.8571	0.7898	0.0270	0.3980
HMS2	5.0000	0.7143	1.0000	0.6657	-0.3333	0.7890
ASB17	6.0000	0.7755	0.5714	0.7444	0.3333	0.2800
LEX3	7.0000	0.7959	0.4286	0.7681	0.5200	0.0060
HMS1	5.0000	0.7245	0.2857	0.6853	0.6522	0.0230
CA425	6.0000	0.6939	0.7143	0.6636	0.0476	0.2820
Mean	5.5294	0.7296	0.7213	0.6926	0.0892	

Table 3. Number of alleles (AN), expected heterozygosity (HE), observed heterozygosity (HO), polymorphic information content (PIC), inbreeding coefficient (F) and deviation from Hardy-Weinberg equilibrium (HWE) at 17 microsatellite loci in KBN group

Marker	A_N	H_E	H_O	PIC	F	HWE
VHL20	8.0000	0.8265	0.8571	0.8058	0.0400	0.8150
HTG4	4.0000	0.6429	0.5714	0.5849	0.1864	0.6240
AHT4	6.0000	0.7347	0.8571	0.7006	-0.0909	0.8260
HMS7	5.0000	0.7041	0.4286	0.6574	0.4545	0.0120
HTG6	5.0000	0.6224	0.5714	0.5874	0.1579	0.2930
AHT5	7.0000	0.8265	1.0000	0.8033	-0.1351	0.8150
HMS6	7.0000	0.8163	0.7143	0.7923	0.2000	0.0240
ASB23	7.0000	0.8469	0.4286	0.8277	0.5500	0.0060
ASB2	8.0000	0.8367	1.0000	0.8165	-0.1200	0.7510
HTG10	7.0000	0.7653	0.4286	0.7333	0.5000	0.0050
HTG7	5.0000	0.7755	0.5714	0.7397	0.3333	0.0310
HMS3	6.0000	0.7755	0.5714	0.7444	0.3333	0.0660
HMS2	5.0000	0.7245	0.5714	0.6853	0.2836	0.1980
ASB17	7.0000	0.8163	0.7143	0.7923	0.2000	0.0960
LEX3	6.0000	0.7755	0.4286	0.7444	0.5068	0.0060
HMS1	4.0000	0.6429	0.4286	0.5849	0.4000	0.1290
CA425	5.0000	0.5510	0.7143	0.5207	-0.2245	1.0000
Mean	6.0000	0.7461	0.6387	0.7130	0.2185	

number of alleles was 4.9412 and varied from 3 (HMS7, HMS1) to 7 (VHL20, AHT5, ASB17). The observed heterozygosity ranged from 0.3333 (HMS7) to 1.000 (VHL20, AHT5) with mean of 0.7157, while the expected heterozygosity ranged from 0.4861 (HMS7, HMS1) to 0.8333 (VHL20) with mean of 0.6846 (Table 1). The mean number of alleles in KBA group was 5.5294, varied from 3 (HTG6) to 7 (AHT5, ASB2, LEX3). The observed heterozygosity ranged from 0.2857 (ASB23, HMS1) to 1.000 (HTG4, AHT5, HMS2) with mean of 0.7213, while the expected heterozygosity ranged from 0.4388 (HTG6) to 0.8469 (AHT5) with mean of 0.7296 (Table 2). In KBN group the mean number of alleles was 6 and varied from 4 (HTG4, HMS1) to 8 (VHL20, ASB2). The observed heterozygosity ranged from 0.4516 (HTG6) to 0.8548 (ASB2) with mean of 0.6387 while the expected heterozygosity ranged from 0.4286 (HMS7, ASB23, HTG10, LEX3, HMS1) to 0.8469 (ASB23) with mean of 0.7461 (Table 3).

Our data of microsatellite diversity are consistent with the data from previous studies. Indicators of microsatellite diversity reported in the literature for other horse breeds mostly ranged from 3.3 to 10.7 for number of alleles, from 0.45 to 0.78 for HO and from 0.47 to 0.82 for HE (Canon et al., 2000; Aberle et al., 2004; Galov et al., 2005; Solis et al., 2005; Plante et al., 2007; Di Stasio et al., 2008; Giacomoni et al., 2008; Shasavarani and Rahimi-Mianji, 2010). Based on all the observed parameters, in KBR and KBN groups VHL20 locus showed the highest microsatellite diversity. Locus HMS7 was the least diverse in KBR group, while HMS1 locus was the least diverse in KBN group. In KBA group the highest microsatellite diversity showed AHT5 locus and HTG6 locus was the least diverse. For the observed

heterozygosity, in KBR group values for VHL20 and AHT5 loci reached the maximum level (i.e. Ho = 1), in KBA group values for HTG4, AHT5 and HMS2 loci reached the maximum level and in KBN group values for AHT5 and ASB2 loci reached the maximum level. This result indicates that the studied populations originated from the appropriate number of parent generations. The average HE in all investigated groups indicated the existence of high genetic variability in populations of crossbred horses in B&H.

The greatest differences between HO and HE in our study were observed for LEX3 (KBR group) and ASB23 (KBA and KBN groups) loci. The same loci showed the highest inbreeding coefficient, the highest deviation from HWE and substantial heterozygote deficit. According to Galov et al. (2013) highly significant deviation from HWE combined with substantial heterozygote deficit is likely to indicate locus-specific genotyping problem due to null alleles.
The largest disproportion between observed and expected heterozygosity was found in KBN group. According to Berber et al. (2014) larger disproportion between observed and expected heterozygosity could be an indicator of within-population inbreeding or conversely population subdivision reduction.

In population genetic analysis, genetic markers with PIC values higher than 0.5 are normally considered to be informative (Shasavarani and Rahimi-Mianji, 2010). The PIC values, detected in our study, suggested that most of the markers were quite informative (PIC > 0.5) in terms of their suitability for genetic diversity studies.
An increased inbreeding coefficient was detected in KBN group (0.2185). High level of inbreeding coefficient in KBN group could be due to small sample size. Inbreeding coefficient values for KBR and KBA groups (0.0458, 0.0892, respectively) indicate no shortage of heterozygotes. Deviation from HWE in KBR group was found in one locus, in KBA group in three loci. In KBN group deviation from HWE was found in six loci. Possible causes for disequilibrium in KBN group were small population size and inbreeding. Overall observed heterozygosity within KBR group is higher then expected, indicating quite large number of heterozygotes probably due to expressed different genetic variants from parental breeds. This is not a case in KBA and KBN groups.

The numbers of detected alleles are within the range for Arabian horse (Rukavina et al., 2015a) and thoroughbred horse populations from Bosnia and Herzegovina (Rukavina et al., 2016). On the other hand, heterozygosity levels are higher in these three types of crossbreeds then in "pure" breeds observed in abovementioned studies.

Conclusions

The results of the present study suggest that the investigated populations of crossbred horses raised in B&H are not affected by major loss of genetic diversity. The applied set of 17 microsatellite markers proved to be specific enough for use in study of genetic structure of crossbred horses. An increase in inbreeding coefficient and sufficient heterogenity between animals in KBN group indicate occurrence of consanguineous mating. The present research contributes to the knowledge of population structure and current status of genetic structure of the investigated populations. Also, the results offer basic information that may be helpful to horse breeders in designing and managing future breeding strategies.

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VALIDATION OF VAGINAL SELF-SAMPLING AS AN ALTERNATIVE OPTION IN PCR BASED DETECTION OF HPV IN CERVICAL CANCER SCREENING IN BOSNIA AND HERZEGOVINA

Ksenija Radić[1], Lejla Pojskić[1*], Anja Tomić-Čiča[2], Jasmin Ramić[1], Daria Ler[3], Naida Lojo-Kadrić[1], Naris Pojskić[1], Kasim Bajrović[1]

[1]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*
[2]*Femina, Private gynaecological practice, Sarajevo, Bosnia and Herzegovina*
[3]*International University of Sarajevo, Sarajevo, Bosnia and Herzegovina*

***Correspondence**
E-mail: lejla.pojskic@ingeb.unsa.ba

Received
January, 2017
Accepted
April, 2017
Published
June, 2017

Research article

Abstract

Cervical cancer represents a serious health problem affecting women worldwide especially in developing countries due to low socioeconomic status, inadequate health-care infrastructure, weaknesses in education on this particular issue and lack of effective screening programmes. The primary aim of this study was to assess alternative screening method for the improvement of cervical cancer prevention in conditions of Bosnia and Herzegovina (B&H), which could be applicable in other developing countries as well. The study was conducted on 101 subjects who provided their self-sampled vaginal swabs and/or cervical specimens collected by their gynecologists. Universal Human Papilloma Virus (HPV) primer set optimized to detect a wide range of HPV types was used for HPV genotyping from obtained swab samples in multiplex PCR. Amplicons were analyzed in agarose gel and *Agilent 2100* bioanalyzer – a platform based on microfluid technology. Inter-rater agreement *kappa* (MedCalc2) was used to assess concordance between results of cervical and vaginal sample analysis. Out of 39 subjects who provided their vaginal and cervical samples, results of HPV detection mismatched in 10% of the cases. *Inter-rater agreement* showed good strength of coincidence between the results of cervical and vaginal sample analysis (*kappa*=0.748, CI=95 %). We presented an alternative PCR method for the detection of HPV based on vaginal self sampling which is affordable, informative, simple and applicable with high coverage level of defined targeted population and potentially significant in the given cultural and socioeconomic context.

Key words: *HPV, cervical cancer, PCR, screening, self-sampling*

Introduction

In spite of the advancement and rapid improvement of technologies in the fields of medicine, molecular biology and genetics, cervical cancer still represents a serious health problem affecting women worldwide and causing death of approximately 250000 women every year (WHO, 2007). According to the data of World Health Organization from year 2002 (WHO, 2002), about half a million new cases

are diagnosed each year, 80 % of which are recorded in developing countries. Nobel laurate Harald zur Hausen was the first to discover that the cause of cervical cancer lies in persistent infection with oncogenic types of the human papillomavirus (HPV) (Boshart et al., 1984).

Human papilloma virus (HPV) is type of small DNA virus which infects epithelial tissue of higher vertebrates leading to cell proliferation, which often results in the formation of papillomas. Human papillomaviruses are species specific. So far, over a hundred types of HPV have been identified and most of them infect anogenital system; those were the focus of this research. Infection with these HPV types is often asymptomatic, which makes regular gynecologic examinations crucial in the detection of possible precancerous and cancerous lesions caused by this virus. Protein L2 is a structural molecule of HPV and has many important roles: it introduces HPV DNA in viral particles formed by self-assembling of L1 main structural protein (Munger et al., 2004; Zhou et al., 2004); it helps virus enter the epithelial cells; it incorporates viral components in nucleus and also represents a potential target for new generation of protective vaccines (Pereira et al., 2009; Peter et al., 2013).

HPV genome covalently binds to mitotic chromosomes (You, 2010) and thus ensures its transfer and distribution to daughter cells. Although HPV genome is usually found in episomal form in the infected cells, it can also be inserted into genome of the cell and viral integration seems to be a crucial step in progression of low-grade to high-grade cervical intraepithelial lesions and cervical carcinoma (Kalantari et al., 1998; Klaes et al., 1999). This can explain the strong association between some types of HPV and high-grade cervical lesions or cervical carcinoma. Transcripts of integrated genomes are more stable and, in case of HPV 16 it has been shown that infected cells with the integrated HPV genomes had selective advantage in their growth (Jeon et al., 1995). Epigenetic factors associated with the progression of HPV infection from subclinical stage to invasive carcinoma are being investigated as well. Demethylation of CpG region happens before or at the same time with neoplastic progression (Badal et al., 2003) and it is probable that methylation pattern changes during replication cycle of HPV.

Identifying the type of HPV has a clinical significance with respect to different pathogenic potentials of various types. It has also been shown that the distribution of different HPV types varies in different geographic regions (Mammas et al., 2008), so knowledge of the frequencies of certain HPV types is important in creating appropriate screening strategies and policies of cervical cancer screening and prevention. Bosnia and Herzegovina, as many other developing countries, has no developed strategies of organized screening. Cervical carcinoma is the second most common carcinoma which affects women in Federation of Bosnia and Herzegovina with rate 6.9 per 100000 inhabitants for malignant neoplasms of cervix in 2011 (ZZJZ FBIH, 2011). Developing countries have a huge problem with cervical carcinoma because of factors such as: low socioeconomic status, limited health infrastructure, specific cultural context, inadequate health-care and lack of effective screening strategies. World Health Organization particularly points out the importance of well-organized screening in cervical carcinoma prevention. In the development of effective screening strategies special attention should be paid to their good integration into the existing health system, having in mind specific social, economic and cultural conditions in developing countries.

For all of the above-mentioned reasons, this research focused on the development of

alternative methods for HPV detection which could be potentially applicable for screening of women in developing countries. The primary aim of the study was optimization of the HPV detection based on the PCR methods with the special emphasis on the screening from self-sampled vaginal swabs. Multiplex PCR method was also developed to genotype some of the most common HPV genotypes in European population.

Materials and methods

In this study, the participants were volunteers who signed informed consent positively evaluated by Scientific Council of research institute where the study was conducted. The participants also provided relevant information for this study before biological sampling in the form of questionnaire, which contained no personal information.

The total of 101 female individuals accepted participation in study during the study period of two years (2009-2011). The sample was geographically limited to the Canton Sarajevo and divided into two groups:

1) general population (75 subjects who were randomly chosen; contacted in their firms/institutions or at home addresses),
2) special population (26 subjects addressed by gynecological consulting rooms because of the presence of certain cytological abnormalities or positive history of HPV infection).

Two methods of gynecological sampling were conducted in this study: self-sampling of vaginal swabs and sampling of cervical swabs provided by medical professional. Examinees were given detailed oral and written instructions on self-sampling. All sample swabs were taken using *DNAPapTM Cervical SamplerTM* (*QIAGEN GmbH*, Germany). DNA was isolated from all the collected cervical and vaginal samples using simple salting-out procedure (Miller et al., 1988). Agarose gel-electrophoresis was used for qualitative assessment of the obtained DNA. Concentration of the obtained DNA was determined by spectrophotometry (UV mini 1240, Shimadzu, Japan). Control PCR was performed with universal HPV primers as described before (Maki et al., 1991) which can detect a broad spectrum of HPV types yielding the product of around 270bp. The amplification reaction conditions were: 10% PCR buffer and 1 unit of Taq DNA Polymerase per reaction (TrueStart Taq Polymerase and Buffer, *ThermoScientific*, USA), 25mM MgCl2 (Fermentas, Latvia), 50 pM each of forward and reverse universal primer (Biotez GmbH, Germany). Thermal conditions of PCR were 30 cycles of chain reaction of denaturation step at 95°C for 60 s, annealing at 34°C for 45 s and elongation at 72°C for 45 s, after single initial denaturation step at 95°C for 5 min and followed by single extension step at 72°C for 5 min. PCR reactions were successfully optimized to the volume of 10 μl.

In addition to the optimization of universal marker PCR, multiplex PCR was optimized as well, in order to screen for the most common HPV types in European population further into the experiment. Positive controls for PCR reaction were plasmids with integrated HPV sequences and previously genotyped samples in standardized laboratory (*Genetic Lab*, Romania). Negative controls included validated HPV negative sample and blind control (water). Since we included two additional primer sets for detection of HPV types 31 and 35, amplification reaction was optimized with respect to the annealing temperature and concentrations of the chemicals and template.

Six primer pairs (Karlsen et al., 1996) were included in simultaneous multilocus co-amplification reaction to detect for HPV types

11, 16, 16 a, 18, 31 and 35 complementary templates. Primer pairs were divided in two reactions: (I) types 11, 16, 16a and (II) types 18, 31, 35 in order to avoid possible mispriming and unspecific products.

PCR reaction was done in total volume of 10 µl using 2 µl of DNA template and 20pM oligonucleotide sequences from three primer pairs in each primer set –set A (sequence specific primers for HPV types 11, 16 and 16a) and set B (sequence specific primers for HPV types 18, 31 and 35). A touch-down PCR technique was used to obtain high specificity of annealing conditions for primer pairs in multiplex reaction as follows: initial denaturation at 94°C for 3 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s, then 20 repetitions of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 45 s, completed with final extension at 72°C for 7 min.

A platform based on microfluid technology (*Agilent 2100*, *Agilent Technologies*, USA), was used for highly precise detection and evaluation of the size of obtained PCR products. Statistical analysis *Inter-rater agreement* (*kappa*) of collected data was done using MedCalc2 statistical software.

Results and discussion

Swab samples were collected from total of 101 subjects aged 20-60 from Canton Sarajevo. DNA was successfully isolated from 98 individuals which represent 98% of the samples. It was not possible to obtain DNA from three samples of vaginal swabs.

Of 103 women who were contacted, 101 expressed interest to participate in the study, which reflects high response rate (98%). There were 39 participants who provided both cervical and vaginal samples for the purpose of cross-validation of the results obtained from two different biological specimens (self-sampled and sample taken by medical professional) of the same individual.

The applicability of the plasmids with incorporated sequences of HPV 11, 16, 16a, 18, 31 and 35 used as positive controls has been confirmed with positive PCR signal with respective primers. The reaction for each plasmid was performed in duplicate with accompanying negative control. Figure 1 shows that, besides HPV detection in cervical swabs, it was also possible to detect HPV infection in self-sampled vaginal swabs using universal HPV primer pairs.

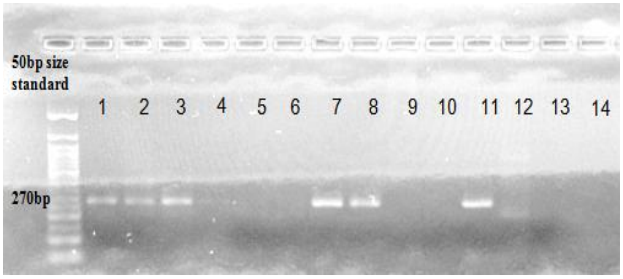

Figure 1. HPV detection in cervical and vaginal samples. DNA ladder (50 bp) in lane 1; lanes 2 to 7 – PCR products of self-sampled vaginal swabs (four HPV positive, two negative); lanes 8 and 9 – cervical and vaginal samples of the HPV positive subject; lanes 10 and 11 – cervical and vaginal samples of the HPV negative subject; lanes 12 and 13 – positive and negative controls previously genotyped by standardized laboratory; lane 14 – PCR negative control

Out of 101 collected vaginal samples we detected amplification of universal HPV sequence in 17 or 17.17% cases. Out of 17 HPV positive samples 11 (11% of overall study sample) had positive signal for high-risk HPV targeted DNA sequence: HPV type 16 in 6 cases, 31 in 2 cases and 35 was positive in one case.

Two specimens were positive for 2 high-risk genotypes- in one case 16 and 35 and in second

case 18 and 31. HPV type 11 was not detected in any of the analyzed samples. This finding also indicates that 65% of vaginal HPV infections are due to high-risk HPV genotypes.

PCR protocol with universal HPV primer modified according to (Maki et al., 1991) could be a valid template to detect targeted HPV genotypes relevant for European population in both cervical and vaginal – self-collected samples (Figure 2). PCR reactions were successfully optimized to the volume of 10 µl using TrueStart Hot Start Taq DNA Polymerase (ThermoScientific, USA) as minimum volume reliable for succesful HPV detection. As the reduction of volume reduces cost by 3-5 x per reaction this is also qute cost-effective approach.

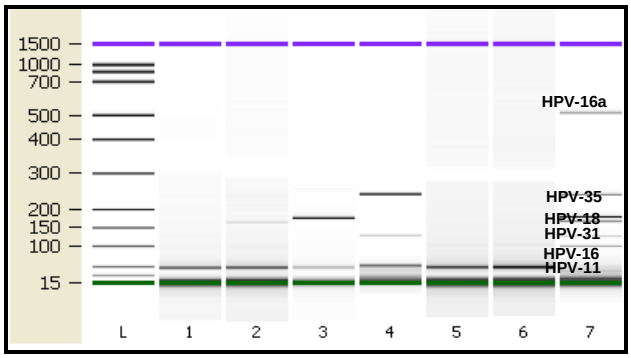

Figure 2. HPV genotyping; L – DNA ladder; lane 1 – HPV negative sample; lane 2 – HPV positive sample, type 31 (153 bp); lane 3 – HPV positive sample, type 18 (172 bp); lane 4 – HPV positive sample, type 35 (230 bp) and 16 (119 bp); lanes 5 and 6 – HPV negative samples; lane 7 – post-PCR mix of plasmid controls (HPV 11 – 80 bp, HPV 16 – 119 bp, HPV 31 – 153, HPV 18 – 172 bp, HPV 35 – 230 bp; HPV 16a – 499 bp)

Out of 39 vaginal samples that had a cervical sample counterpart, 35 (around 90%) showed concordance in the results of cervical and vaginal samples analysis. In four samples, HPV was detected in cervical smears, while it was not possible to detect it in vaginal swabs probably due to low quantities of viral DNA template retrieved during sampling. The quality of the band signals from vaginal swabs varied among the samples. Analysis of the disputed four samples using Real-Time PCR based method showed that the viral infection was present in the vaginal swabs as well, but in the lower quantity, which can be seen from the amplification diagram (Figure 3) where signal of the vaginal swab crosses threshold in the later stages compared to the cervical smear of the same patient.

Inter-rater agreement-kappa (Altman, 1991) of 0.74 (CI=95 %) showed good agreement between the results of cervical and vaginal sample analysis.

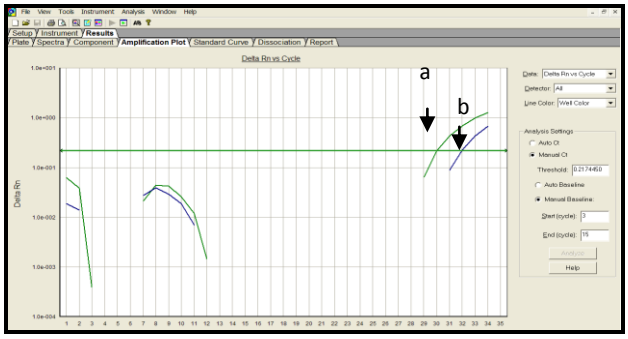

Figure 3. Real Time PCR Amplification Plot – cervical (a) and vaginal (b) sample of the same examinee showing successful PCR amplification in both.

Salting out procedure is a simple, effective and economical method for the extraction of DNA from cervical and vaginal samples. The three samples from which DNA extraction proved impossible serve as a reminder that the subjects should be thoroughly informed on the importance of strict adherence to the instructions on self-sampling. Compared to the standard PCR, Papanicoulaou test (Papa-test), the golden standard in detection of precancerous changes, has lower sensitivity (61.3%) with wider dispersion interval (18.6-94%); sensitivity of PCR is far greater (90%), with more compact interval (84.9-100%) (De Guglielmo et al., 2010) which does not change with the age of patients. However, the clinical importance of such high PCR sensitivity in identification of women with the increased risk for developing

cervical cancer (women who already have CIN2) is questionable and should be discussed. PCR specificity is lower, because it detects also transient HPV infections which are so common among the younger population.

Since previous studies have already confirmed high concordance rate between PCR and other sequence based detection - Hybrid Capture (HC) test (Soderlund-Strand et al., 2005), the multiplex PCR method described here could be validated in small-scale setting for the purpose of evaluation of reliability of multiloci detection sensitivity and specificity prior to further application.

Nevertheless, it should be emphasized that: 1) the link between viral load and progression toward cervical carcinoma is still examined; 2) in socioeconomically weak areas, especially rural, some women rarely have a chance for screening (Peter et al., 2013; Chou et al., 2016). Having this in mind, HPV detection using less costly methods can serve as an early indication and warning and also motivate women to inform themselves about this topic, pay attention to their life-style and improve their overall health (Arbyn et al., 2015). Combination of cytology and molecular-genetic methods can have very significant, high predictive value for cervical cancer (98%) which can decrease screening intervals for HPV negative patients and thus contribute to cost reduction for health-care programmes (De Guglielmo et al., 2010; Zehbe et al., 2011; Vassilakos, 2016).

It was shown here that the universal HPV primer pair can detect genotypes 31 and 35 as well. Some of the HPV positive samples could not be genotyped with the HPV specific primers used in this study which corresponds with the assumption that universal HPV primer pair can detect wide range of HPV genotypes.

The fact that it was not possible to genotype HPV from 35% of HPV positive examinees could indicate the presence of more relatively common HPV types in population of Bosnia and Herzegovina. The most common type detected in this study was HPV 16, but having in mind that the sample was geographically limited it cannot be concluded that this is the most common HPV type in Bosnia and Herzegovina. Since there is no official data for Bosnia and Herzegovina, there is a need to conduct a serious, large population study which would provide a basis for taking the appropriate preventive measures.

The discordance in results of four cases of examined cervical and vaginal samples can be explained by the lower amount of viral template in vaginal swab since the highest concentration of viral particles is in transformation zone of the cervix.

The potential use of self-sampled vaginal samples analysis for screening is concordant with the earlier findings (Karwalajtys et al., 2006; Petignat et al., 2007). In every study which examined the acceptance of self-sampling, women prefered self-sampling (Bidus et al., 2005) – it is far less uncomfortable and less embarrassing (Petignat et al., 2007). Analysis of self-sampled vaginal swabs for HPV detection could encourage women to participate in screening programmes. This could be particularly significant for women who do not regularly attend gynecological examinations for different reasons. The test could play role in early detection of pathological changes of cervix. Lower specificity of PCR test could be overcome by increasing combination of direct HPV detection and standard cytological procedures or by applying this test to women older than 35 (when prevalence of HPV positive infections decreases and prevalence of cervical intraepithelial neoplasias increases). While standard diagnostic kits mostly require high technical, financial and logistic support, this test is affordable and could be significant in

screening strategies of developing countries, but should be validated against the golden standard and validated diagnostic kits. In that sense, tests based on PCR technology could represent more simple and economic alternative in screening for cervical cancer prevention. It could be particularly important in regions which lack adequate infrastructure and cytologic analysis of high quality and critical areas where women do not have approach to health-care system or, for some cultural or traditional reasons, do not go to gynecological examinations.

For efficient screening programme it is necessary to: define targeted population and insure high rate of its coverage, offer health-care services of high quality which will enable identification, treatment and monitoring of the patients, provide trainings for health-care workers, inform and educate women and develop strategies which can be incorporated into national programme for tumor prevention.

There are several criteria which need to be fulfilled in order to have a successful screening based on HPV testing (WHO, 2012). The applicability of the method used in this study will be examined with regards to the given criteria:

1) minimally invasive sampling – since it has been shown that the virus can be detected in self-samples vaginal swabs, self-sampling could be applied as the minimally invasive procedure which is the least uncomfortable and economical as well.
2) high sensitivity and specificity of method to detect wide range of genital HPV types – although sensitivity and specificity of this particular test should be validated against standard diagnostic procedures, it has already been shown that PCR itself is highly sensitive method. Sensitivity can be increased by amplification of internal control (such as beta-globin gene) which could enable detection of potential PCR inhibition. Lower specificity, which is the main disadvantage of HPV PCR test could be overcome by using this test for screening women older than 30-35. The main advantage of the test with universal HPV primer pair is possible detection of wide spectrum of genital HPV types.
3) high level of intra- and interlaboratory reproducibility – reproducibility of the test can be checked by repeating the experiment and comparing results with other laboratories. Interlaboratory reproducibility in this study was checked by comparison of several samples with partner laboratory Genetic Lab (Romania). Additional three samples were analyzed by HC II test at the Clinical Centre University of Sarajevo. The complete concordance of the results was obtained, although the number of compared samples was limited. Functionality of the method was examined by repeating experiments in the laboratory – series of samples were analyzed under the same, controlled experimental conditions, with controls.
4) suitability of the method for automatic procedures and reading – PCR is automated and widely used method. Agilent Bioanalyzer also provides quick and precise analysis of given PCR products.
5) possibility to analyze large number of samples – this considers the possibility of collecting large number of samples and the existence of capacities for their analysis. This study indicates that there is a possibility that women actively take part in the screening programmes through self-sampling. It is necessary to standardize the optimized PCR method for HPV detection on the state level, to provide training of the staff and to improve effectiveness of existing laboratories and available human and material capacities.

There are potential capacities to apply this method but screening strategies need to incorporate timely and planning measures for effective usage of the available capacities on the state level.

6) short testing time – in this research, it is empirically estimated that the analysis of 20-25 samples, on average, takes 2 days per one analyst. The testing time could be improved through the distribution of work within the team.

7) economy – molecular-genetic HPV testing within the screening programme could quickly address HPV positive women to colposcopy in order to detect precancerous and cancerous lesions in time. HPV negative women would have longer screening intervals which would reduce the costs to health-care system. Hybrid Capture tests are validated but technically and financially demanding for developing countries. According to the study conducted in Brasil, HPV testing costs with Hybrid Capture technology are 3.84 times higher compared to PCR method (Nomelini et al., 2007). Detection of HPV based on PCR is more accessible, affordable, sensitive and simple method with high negative predictive value and, according to some studies (Morris, 2005; Nomelini et al., 2007; Romero-Pastrana, 2015) it will be the best option for primary screening.

Conclusions

Based on our research results, it is possible to successfully isolate DNA from cervical and vaginal samples using Miller's salting-out procedure. The modified PCR method with universal HPV primer presented here is sensitive to the level required to detect HPV infection presence in both cervical and vaginal samples. Having in mind that most of the developed molecular-genetic tests require high technical, financial and logistic support, detection of HPV based on PCR technology is simple, highly sensitive method with high negative predictive value which is also more affordable so it could represent a good alternative for screening in developing countries. Validated and clinically most confident method, previously approved by the relevant institutions, should be chosen for this purpose. *Kappa* value recorded in this study (0.784; CI=95%) indicates good agreement between the results of cervical and vaginal samples analysis. The optimized PCR method with universal HPV primer enables HPV detection in self-sampled vaginal swabs. This is particularly important for screening in developing countries. Analysis of self-sampled vaginal swabs for HPV detection could motivate women to participate in the screening programs which would be extremely important in the prevention of cervical cancer.

Acknowledgements

Authors would like to express gratitude to all the participants in the study.

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SCREENING FOR GMO IN FERMENTED SOY SAUCE

Anesa Ahatović[1*], Edina Ljekperić[2], Mirza Nuhanović[2], Adaleta Durmić-Pašić[1]

[1]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*
[2]*University of Sarajevo, Faculty of Science, Sarajevo, Bosnia and Herzegovina*

***Correspondence**
E-mail: anesa.ahatovic@ingeb.unsa.ba

Received
March, 2017
Accepted
April, 2017
Published
June, 2017

Short communication

Abstract

Soy sauce is worldwide popular condiment of Asian origin. With the advent of GM soybean production, soy sauce drew the interest of food safety control. Samples collected for inspection are generally of industrial grade soy sauce type, which is produced from hydrolyzed soybean and grain. Following the failure to perform RealTime PCR based GMO screening on a number of submitted samples we tested our screening system on soy sauce produced following traditional method based on fermentation. Four batches of soy sauce were produced and DNA extracted. DNA concentration ranged from 32.68 to 65.36 ng/µl. Amplification of taxon specific target was successful with rather high Ct (> 30). Promoter P-35S sequence was not detected, but T-NOS was detected in three samples with values reaching or exceeding LOD of the method. The results show that it is possible to detect transgenic elements in traditionally produced soy sauce while DNA extraction from industrial grade soy sauce is not possible.

Key words: *Soy sauce, DNA extraction, RealTime PCR, GMO*

Introduction

Soy sauce is an Asian condiment that originated in China about 2500 years ago. Its use was later spread to the other parts of Asia while, it was brought to Europe in the 17th century. Its recognition in the Western societies followed the rise in popularity of Asian cuisine. Traditional soy sauce is mature, fermented product, made from soybeans, grains and salt. The production process is slow and does not achieve industrial scale. As current cumulative consumption greatly surpasses the capacities of traditional production, industrial grade soy sauce is produced from hydrolyzed soy protein and grain. While this process results in affordable product of satisfactory sensory qualities, acid hydrolyses renders this type of product inaccessible for DNA based analysis such as those applied in food safety sector. With the increasing presence of GM soybean in food supply, soy sauce became an object of scrutiny from food safety perspective.

According to ISAAA report 83% (92.1 million hectares) of the 111 million hectares of the globally planted soy are biotech (James, 2015). Legal framework on GMO of the European Union and Law on GMO in Bosnia and Herzegovina (2009) require labeling of products

that contain more than 0.9 % GM content per species. Successful implementation of any legal framework depends on the abilities of analytical laboratories to provide correct information. Therefore, official laboratories utilize validated analytical methods and DNA based methods are the methods of choice when it comes to GMO analysis (Anklam et al., 2002). Unfortunately, validation is generally performed on general type of matrix such as grain or flour and does not guarantee comparable performance on different type of matrix. Thus, when DNA extraction from industrial grade soy sauce proved to be challenging, we decided to explore the matrix rather than alter the method. We hypothesized that the DNA extraction method validated for soybean would provide sufficient quantity of PCR grade DNA from soy sauce produced by traditional method.

Materials and methods

We prepared soy sauce by traditional method in order to test the capacity of CTAB DNA extraction procedure validated for soybean to yield sufficient quantity of PCR grade DNA.

The production method was derived by analyzing a number of traditional recipes and extrapolating common ingredients and steps. All ingredients were procured locally. Soybean, being the target ingredient, was obtained from different manufacturers (Tab. 1). Raw soybeans were soaked in water for 15 hours and then boiled for about an hour. Cooked and smashed soybeans were mixed with ca. half the amount of wheat flour to form a homogeneous mixture. The obtained mixture was divided in smaller cakes and left for about 20 days in a warm and humid place and allowed for mold to develop. Following the development of mold, the cakes were dried in a warm place and soaked in salt brine (6 % NaCl solution). Fermentation was allowed to proceed slowly with occasional agitation. The fully fermented mixture was filtered to remove the solids while liquid soy sauce was used for DNA extraction and GMO screening.

Table 1. List of analyzed samples

Sample ID	Country of origin	GMO label
1/16	Abu Dhabi, United Arab Emirates	-
2/16	China	-
3/16	Netherlands	GMO free
4/16	Bulgaria	-

Genomic DNA was extracted from 2 ml of soy sauce using CTAB precipitation method validated and recommended by the European Union Reference Laboratory for GM Food and Feed (EN ISO 21571: 2005. Annex A, par. A3). The quality and quantity of extracted DNA was measured by UV spectrophotometry (UV-VIS Spectrophotometer, Shimadzu, Japan).

To test if the obtained DNA extracts contain amplifiable DNA we used RealTime PCR amplification of lectin ie. taxon specific target for soy (EURL-GMFF & ENGL, 2010). GMO screening was performed using RealTime PCR methods which target most common elements of transgene construct – cauliflower mosaic virus promoter (p-35S) and nopaline synthase gene terminator from Agrobacterium tumefaciens (t-NOS).

These methods are validated by the Italian National Reference Laboratory for GMO (IZSLT, 2011). RealTime PCR reactions were carried out in an ABI 7300 RealTime PCR instrument (*Applied Biosystems*). Certified reference materials by JRC-IRMM were analyzed parallel to samples and used as positive controls. The raw data were analyzed using Sequence Detection Software (Applied Biosystems).

Results and discussion

Following traditional recipe we prepared four batches of soy sauce originating from different sources. Some physical and chemical characteristics of the obtained soy sauces are listed in Tab. 2. While soy sauces made by industrial procedure have liquid consistency, dark color and sour or salty flavor our traditional products have thicker consistency and lighter color.

Table 2. Physical and chemical characteristics of soy sauces produced following the traditional recipe

Sample ID	Color	Consistency	Flavor	pH value
1/16	Light brown	Mash	Sour, salty	4.49
2/16	Light brown	Mash	Sour, salty	3.70
3/16	Light brown	Mash	Sour, salty	3.80
4/16	Dark brown	Mash	Sour, salty	3.90

Genomic DNA was successfully extracted from all samples. Absorbance at 260 nm, 280 nm and 230 nm showed sufficient purity and quantity of DNA extracts for further analysis. However, A260/A230 ratio showed carbohydrates residues which were expected, considering the type of starting material. DNA concentration ranged from 32.68 to 65.36 ng/µl.

Taxon specific target was amplified in all analyzed samples. Even though the amplicons were detected in all samples Ct values were quite high especially compared to CRM Ct value. Despite approximate DNA concentration of 2/16-4/16 samples Ct values varied. Sample 1/16 which showed the lowest DNA concentration (32.68 ng/µl) had lower Ct value compared to 3/16 and 4/16 (40 ng/µl). However, if we consider that the total DNA also included DNA of lactobacilli, the discrepancy in DNA concentration and Ct values ratio is not unexpected. Sample 1/16 also had the highest pH value, indicating less advanced fermentation.

High Ct values for lectine (30.69-34.53) indicated low concentration of target DNA but still within limit of detection (LOD) of the method. All samples tested negative for p-35S, while 3 samples tested positive for t-NOS. High Ct values for t-NOS reactions (34.49-36.30) indicated that concentration of the target DNA approaches LOD of the method (LOD≥4C). Considering LOD of the t-NOS method only sample 1/16 tested positive.

According to high Ct values for lectine we can conclude that concentration of target DNA was low which reflected to Ct values for t-NOS. Additional optimization of DNA extraction protocol such as adjustment of amount of the starting material and additional purification step with chloroform are necessary to increase target DNA yield.

Table 3. Ct values of RealTime PCR reactions

Sample ID	Lectine	p-35S	t-NOS
1/16	32.58	-	34.49
2/16	30.69	-	35.28
3/16	34.53	-	-
4/16	34.50	-	36.30
10 % CRM	18.26	-	20.38

Previous analyses of industrial soy sauces in our laboratory were unsuccessful regardless of used DNA extraction protocol. Industrial soy sauce is

made from acid-hydrolyzed soy protein. It is assumed that unssuccessful DNA extraction is a consequence of absence of target DNA in the substrate because it is also subjected to acid hydrolysis. Amplification of lectin gene in DNA extracted from soy sauce made following traditional recipe once again confirmed that industrial soy sauce is not suitable for GMO screening using PCR methods.

Conclusions

Although DNA was successfully extracted from traditional soy sauce low DNA concentration and high Ct values for lectine (30.69-34.53) indicated the necessity of additional optimization of DNA extraction procedure. All analyzed samples tested negative for p-35S, while t-NOS was detected in three samples. However, high Ct values for t-NOS reactions (34.49-36.30) indicated that concentration of the target DNA approaches LOD of the method which is bellow the labeling threshold and only sample 1/16 can be considered as positive. The results show that it is possible to detect transgenic elements in soy sauce made following traditional recipe while DNA extraction from industrial soy sauce is not possible.

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IN VITRO ANALYSIS OF TARTRAZINE GENOTOXICITY AND CYTOTOXICITY

Anja Haverić[1*], Damira Inajetović[2], Aneta Vareškić[2], Maida Hadžić[1], Sanin Haverić[1]

[1]*University of Sarajevo, Institute for Genetic Engineering and Biotechnology (INGEB), Sarajevo, B&H*
[2]*University of Sarajevo, Faculty of Science, Sarajevo, Bosnia and Herzegovina*

***Correspondence**
E-mail: anja.haveric@ingeb.unsa.ba

Received
January, 2017
Accepted
April, 2017
Published
June, 2017

Research article

Abstract

Tartrazine (E 102) is widely used yellow food colorant. It is used in nonalcoholic and sports drinks, spicy chips, jams, jelly and chewing gum and also found in many non-food products like soaps, cosmetics, shampoo, vitamins and some drugs. Tartrazine belongs to the most important and diverse group of synthetic dyes – azo dyes. Their use often creates controversies in the public since some of them are toxic, carcinogenic, mutagenic and cause different disorders or allergic reactions. In this study we aimed to evaluate genotoxic potential of tartrazine in human lymphocytes culture and its cytotoxic potential in human lymphocytes and melanoma GR-M cell line. For testing of its genotoxic and cytotoxic potential in human lymphocyte culture, we used chromosome aberration analysis and cytokinesis-block micronucleus cytome assay. For the analysis of its cytotoxic potential in human melanoma cell culture, we applied trypan blue exclusion assay.

Key words: *chromosome aberrations, micronuclei, trypan blue, human cell culture*

Introduction

Synthetic food dyes are extensively used in many aspects of human life. The beginning of synthetic dyes production dates back to the middle of the 19th century and the use of natural dyes is completely overshadowed. Implementation of new analyses in the 20th century revealed significant negative and harmful impacts of certain food dyes on human health thus inducing restrictions on their use. Aside from their negative, mutagenic and carcinogenic, impact on health, synthetic dyes from household or industry also have negative impact on the environment even at very low concentrations (Forgacs et al, 2004; Hosseini et al., 2011). Establishing of appropriate mechanisms for the detection and monitoring of food dyes negative effects on human health is essential to define adequate protection and prevention measures.

Although exacerbating effects of tartrazin in chronic urticaria and asthma sufferers are long known (Lockey, 1977), tartrazine (E 102) is widely used yellow food colorant (Mittal et al., 2007, Saxena and Sharma, 2015). Tartrazine is used in nonalcoholic and sports drinks, spicy chips, jams, jelly and chewing gum, bakery goods, cereals, candies, gelatin and numerous other commodities (Saxena and Sharma, 2015). It is also found in non-food products like soaps,

cosmetics, shampoos, vitamin supplements and some drugs (Amin et al., 2010). In many countries tartrazine is used in catering as an alternative to saffron (Mehedi et al., 2009). Tartrazine belongs to the most important and diverse group of synthetic dyes – azo dyes, which includes about 3000 different compounds. They are widely used and distributed in the environment due to their convenient and inexpensive synthesis, stability and wide range of shades in comparison to natural dyes (Saratale et al., 2011). Use of azo dyes often causes controversies in the public since some of them are toxic, carcinogenic, mutagenic and cause different disorders (Saxena and Sharma, 2015) or allergic reactions in the organism (Bhatia, 2000; Ardren and Ram, 2001; Bourrier, 2006). Studies have also linked the ingestion of dyes (mostly azo dyes) in candies and drinks with hyperactivity and other disruptive behavior in children (McCann et al., 2007).

In this study we aimed to evaluate genotoxic potential of tartrazine in human lymphocytes culture and its cytotoxic potential in human lymphocytes and melanoma GR-M cell line.

Materials and methods

Lymphocyte culture

Peripheral blood was collected into heparinized vacutainers from a healthy female volunteer who signed informed consent form. Lymphocyte cultures were induced in 15-mL sterile, plastic tubes with conical base (Isolab GmbH, Wertheim Germany), which contained 5 mL of PB-MAX Karyotyping Medium (GIBCO-Life Technologies, Grand Island, NY, USA) and 400 µl of peripheral blood. Cultures were harvested after 72 hours of lymphocytes cultivation at 37ºC, using standard procedure (hypotonic treatment with 0.075M KCl, triple ethanol-acetic acid fixation, followed by microscope slide preparations and staining in 5% Giemsa).

Tested substance

Tartrazine (E-102) was dissolved in dH2O and added to the cultures in the 25th hour of cultivation to the final concentrations of 2.5, 5 and 10 mM. The concentrations were determined according to literature (Mpountoukas et al., 2010). Negative controls with equivalent volume of dH2O and positive controls with 0.25 µg/mL of mytomicine C (Sigma-Aldrich Co, St Louis, MO, USA) were set up as well.

Cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The analysis of genotoxic, cytotoxic and cytostatic potential of tartrazine was evaluated in human lymphocyte cultures by applying cytokinesis-block micronucleus cytome assay in vitro. In order to block cytokinesis, 4.5 µg/ mL of cytochalasin B (Sigma-Aldrich Co., St Louis, MO, USA) was added to the cultures in the 45th hour of cultivation. Microscopic analysis at 400x magnification using Olympus BX51 microscope (Tokyo, Japan) included observation of 2000 binuclear cells per each treatment and controls, equally divided among two replicates (OECD, 2014). The frequencies of genotoxicity markers: micronuclei, nucleoplasmic bridges and nuclear buds, were determined according to the defined criteria (Fenech, 2007; Fenech et al., 2003). Cytotoxic and cytostatic effects were assessed by calculating nuclear division index (NDI) and nuclear division cytoxicity index (NDCI) (Fenech, 2000). The frequencies of mononuclear, binuclear, trinuclear, and quadrinuclear cells, as well as apoptotic and necrotic cells, were registered in the total number of at least 1000 cells, counted per each treatment and controls, equally divided among two replicates.

Chromosome aberration assay

For the chromosome aberration analysis, lymphocytes were arrested in metaphase by addition of colcemid (0.18 μg/ml), 90 min before cell harvesting. 300 metaphases (46±1 chromosome) were analyzed per treatment and controls, equally divided among three replicates (OECD, 1997) at 1000× magnification. Structural chromosome aberrations were scored and registered according to the International System for Human Cytogenetic Nomenclature - ISCN (Mitelman, 1995).

Melanoma cell line

Human GR-M melanoma cell line (Culture Collections, Public Health England, London, UK, Cat. No. 95032301) was cultured in RPMI 1640 (Gibco-Invitrogen, Grand Island, NY) supplemented with L-glutamine, 10% of fetal bovine serum (FBS) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) in T-25 flasks (NUNC, Rochester, NY) at 37˚C, 5%CO2 atmosphere with 95% humidity. At the beginning of experiment cells were seeded at a density of 1×105 cells, pre-incubated for 24 h and finally incubated with the selected concentrations of tartrazine for another 48 h.

Trypan blue exclusion assay

For the cytotoxicity analysis trypan blue exclusion assay was performed after 48h of incubation of melanoma cell cultures with tartrazine. Each treatment was carried out in triplicate. Cells were harvested by trypsinization and cell viability (%) was determined as [number of viable cells / (number of viable + non-viable cells)] x 100.

Statistical analysis

Differences in observed frequencies of genotoxic and cytotoxic parameters were calculated using proportion comparison Z-test in WINKS 4.5 Professional Software (TexaSoft, Cedar Hill, TX). Statistical significance threshold was set at 0.05.

Results and discussion

Results of tartrazin genotoxicity analysis, obtained in human lymphocytes cultures and GR-M melanoma cell line appling CBMN Cyt assay, chromosome aberration assay and trypan blue exclusion assay are presented in tables 1-3. Frequencies of genotoxicity biomarkers in CBMN Cyt assay incrased with the increase of tartrazin concentration (Table 1, Figure 1).

Table 1. Results of CBMN Cyt assay in human lymphocyte culture

Treatment	Genotoxicity biomarkers			Cytostasis/cytotoxicity	
	MN	NB	NPB	NDI	NDCI
Negative control	2	0.5	0	1.768	1.752
2.5 mM	1[b]	0[b]	1.5[b]	1.816	1.303
5 mM	2[b]	0.5[b]	6.5[a]	1.584	1.573
10 mM	8[b]	1	8[a]	1.586	1.574
Positive control	65.5	6	8.5	1.308	1.303

Note: MN – micronuclei; **NB** – nuclear buds; **NPB** – nucleoplasmic bridges. Genotoxicity analized on 1000 BN cells per culture, 2 replicate cultures per treatment; Cytostasis/cytotoxicity analized on 500 cells per culture, 2 replicates per treatment. (a) Significantly different against negative control; (b) Significantly different against positive control.

However, comparison with negative controls revealed no significant differences in MN and NB frequencies. In concentrations of 5 mM and 10 mM of tartrazine, significant increase in

frequency of NPB against negative control was detected (p<0.5 for 5 mM; p=0.005 for 10 mM).

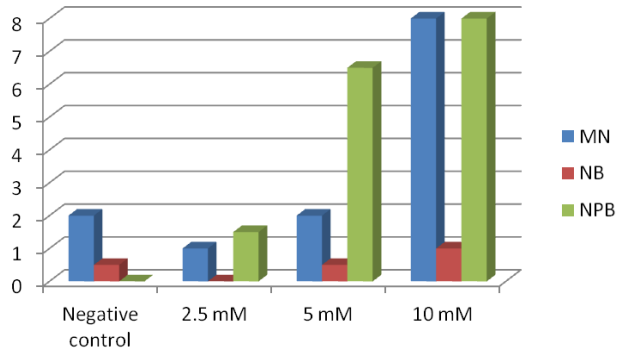

Figure 1. Average frequencies of genotoxicity markers in CBMN Cyt assay expressed per 1000 BN cells in human lymphocyte cultures (negative controls + treatments)

Comparison against positive control were significant for MN frequencies in all three tested concentrations (p=0.0), for NB frequencies in 2.5 mM (p=0.01) and 5 mM (p=0.03) concentrations and for NPB in the lowest tested concentration of 2.5 mM (p=0.026). Neither tartrazine nor mytomicin C induced significant differences in nuclear division indexes (NDI and NDCI) against negative control although nuclear division indexes were decreased in the highest tested concentrations and positive control (Table 1, Figure 2). However, cytotoxic effects of mytomicin C, an antitumor antibiotic, are confirmed in several oral squamous cell lines (HSC-2, HSC-3, HSC-4, Ca9-22 and NA) and human promyelocytic leukemic cell line HL-60 (Sasaki et al., 2006).

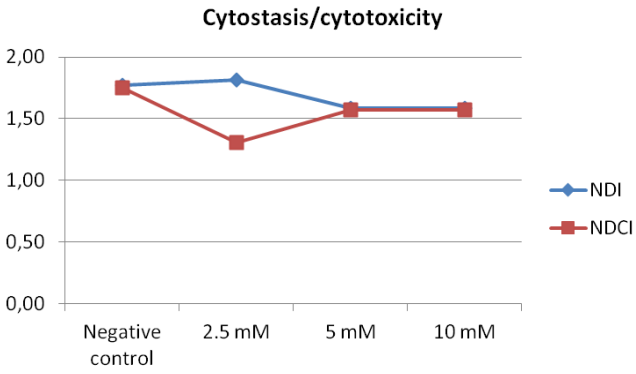

Figure 2. NDI and NDCI values calculated using average frequencies of analyzed cells in cytokinesis blocked human lymphocyte cultures, negative controls and treatments

Proportion comparisson of observed chromosome aberrations revealed the lack of significant differences between each of three tested concentrations and negative control while positive control significantly differed for all tested concentration in chromatid- (breaks and minute fragments) and chromosome-type aberrations (breaks, double minutes) and chromosome rearrengements that were calculated separately (p=0.0). Incidence of pulverzations (pvz) and premature centromere separations (pcs) did not differ between each of three treatments or when compared with positive or negative control (p>0.05).

Previously published data regarding tartrazine genotoxicity are contradictory. Bastaki et al. (2017) dismissed tartrazine genotoxicity in vivo, while Khayyat et al. (2017) reported tartrazine

Table 2. Results of chromosome aberration analysis in human lymphocyte culture

Treatment	chtb, min	chrb, ace, dmin	chre	pvz	pcs
Negative control	0	0	0	0	0
2,5 mM	1	1	0	0	0
5 mM	1	1	0	0	0
10 mM	0.666	1.333	0	0	0.666
Positive control	21	74	24	1	0.333

potencial to induce structural and functional aberrations and genotoxic effects in vivo. Atli-Sekeroglu et al. (2017) also found that tartrazine and its metabolites have genotoxic potential on human lymphocyte cultures both with and without metabolic activation (S9) and can induce cytotoxic effects without S9 and in the highest of concentrations tested (2500 µg/ml). Tartrazin is shown to induce DNA-damage in the gastrointestinal organs at low doses, even at doses approaching the acceptable daily intake (Sasaki et al., 2002) of 7.5 mg/kg/bw/day, which is significant since many products, such as ice creams, desserts, cakes are often marketed without labeling (Elhkim et al., 2007). Study of cytotoxic and genotoxic effects of tartrazine on DNA repair in human lymphocytes demonstrated no cytotoxic effects but revealed significant genotoxic effects in all tested concentrations ranging from 0.25-64.0 mM (Soares et al., 2015), that are far below the concentrations tested in our study (2.5; 5 and 10 mM).

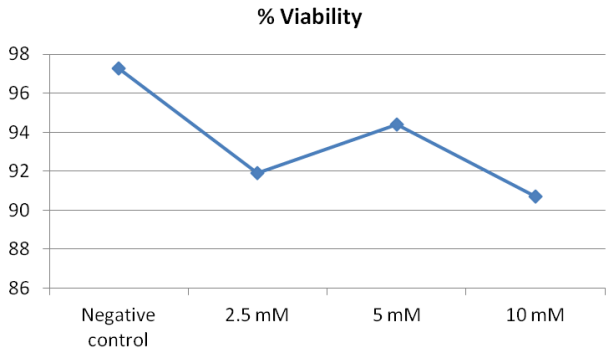

Figure 3. Cytotoxicity analysis using Trypan blue assay in human melanoma GR-M culture

Trypan blue exclusion assay was performed in GR-M human melanoma cell culture in order to estimate tartrazine cytotoxicity. The decrease in cell viability (%) was obvious and significant against negative control (p=0.0), contrasting negligible cytotoxic effects recorded in human lymphocytes culture by CBMN Cyt assay.

Table 3. Results of CBMN Cyt assay in human lymphocyte culture

	Viable	Nonviable	Viability %
Negative control	277	7.75	97.28
2.5 mM	122	10.75	91.90[a]
5 mM	130.5	7.75	94.394[a]
10 mM	124.25	12.75	90.693[a]

Note: (a) Significantly different against negative control.

Overall results suggest that concerns regarding tartrazine use are not unfounded, especially regarding cytotoxicity of tartrazine that should be further evaluated.

Conclusions

Obtained results and conducted statistical analysis show that tartrazine is not genotoxic in human lymphocytes in tested concentrations. Its cytotoxic effects are negligible in human lymphocyte culture but significant in GR-M melanoma cell culture. This finding suggests that future studies of tartrazine should be focused on evaluation of cytotoxic effects using human lymphocytes but also additional cell models, cytotoxicity assays in comparison against cytotoxic effects of other cytotoxicity inducers.

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INFLUENCE OF BASIC VARIABLES ON MICRONUCLEI FREQUENCY AND CHROMOSOMAL ABERRATIONS IN GENERAL POPULATION OF FB&H

Mirela Mačkić-Đurović1*, Izeta Aganović-Mušinović[1], Orhan Lepara[2], Slavka Ibrulj[1]

[1]*University of Sarajevo, Faculty of Medicine, Center for Genetics, Sarajevo, Bosnia and Herzegovina*
[2]*University of Sarajevo, Faculty of Medicine, Department of physiology, Bosnia and Herzegovina*

***Correspondence**
E-mail: mirelamd@yahoo.com

Received
March, 2017
Accepted
May, 2017
Published
June, 2017

Research article

Abstract

The aim of this study was to determine the values of micronuclei (MN) and structural chromosomal aberrations (CA) in peripheral blood lymphocytes from 200 healthy participants of both genders from general population of FB&H, as well as to explore the influence of gender and age on MN and CA frequencies. Standard protocols for MN test, cultivation and micronuclei analysis from peripheral blood binuclear lymphocytes have been applied. MN values ranged from 0 to 8 MN per 1000 binuclear cells. The results suggest that gender and age influence MN frequency, with pronounced effect on 2 MN frequencies. Females on average have higher values of all observed variables of MN test than men. We have also found significant effect of gender – females had increased number of CAs – chromatid type; and of the age in both genders. Frequency distribution of CTAs and CSAs between male and female groups showed predominance of CTAs over CSAs, independently of gender. The results of this study will be incorporated into reference data base for comparative research in future.

Key words: *micronuclei, structural chromosomal aberrations, gender, age*

Introduction

Bio-monitoring is important part of health care for population that is either professionally or environmentally exposed to physical and chemical mutagens and/or carcinogens. It is based on measurement (or) determination of frequencies of different markers that point at the earliest, repairable bioindicators, that appear before the malignancies and/or other diseases (Kopjar et al., 2010). The main postulate for biomarker efficiency is the knowledge of its values in general, unexposed, healthy population.

Micronucleus test (MN) in peripheral blood lymphocytes is one of the most important methods in cytogenetic bio-monitoring. Besides MN-test, analyses of structural chromosomal aberrations (CA) and analysis of sister chromatid exchange (SCE) are important, and those are applied in the surveillance of professionally exposed populations.

CAs include chromosomal breaks and exchanges visible in cells arrested in metaphase-stage They are usually classified into chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs), which can be distinguished morphologically (Collins, 2004).

MN test detects chromosomal aberrations indirectly through nucleus chromatin loses that

lead to MN formation in cell cytoplasm. MN are defined as small, round cytoplasmic bodies, that consist of DNA and are formed during cell division from either acentric chromosomal fragments or whole chromosome that are left behind during anaphase. In vitro MN test is reliable test for the detection of mutagenic factors (Krishnaja and Sharma, 2004). It is also reliable for the detection of genome instability that is related to increased risk of malignancies. Causal relation among increased number of MN and some malignancies and other diseases (diabetes and cardiovascular disease) is evident (Fenech et al., 2011; Andreassi et al., 2011). That justifies the use of MN test as suitable biomarker for long-term estimation of cytogenetic risk among human population.

In estimation of damaging and risk factors on people health, it is extremely important to have a database with biomarkers values measured or determined on large number of individuals from general (healthy) population. Regeneration of database with fresh data is recommended (every two years). According to the leading experts' recommendation, the database should include at least 20 individuals per gender per decade of age (Fenech, 1993). That objective was fulfilled by completion of this study.

The aim of this study was to determine the values of MN test and CA from peripheral blood lymphocytes in 198 healthy individuals from general population of Federation of Bosnia and Herzegovina (FB&H), with the inclusion of both genders.

Materials and methods

Study populations

The study as well as all procedures of blood sampling and handling in laboratory conditions, were performed according to ethical principles and directions for bio-monitoring of human populations. The subjects were informed about the study aim; each subject fulfilled questionnaire with the basic data needed for the study and gave his/her written consent.

The main inclusion criterion was that the subject has not been exposed to physical or chemical agents, has no acute infections or medical exposures to known agents that could interfere with cytogenetic findings. 200 examinees entered the study: 20 per gender per decade of their age. Two men had to be excluded from the study due to previous medical treatment.

Methods

Standard protocol of MN test for cultivation and micronuclei analysis from peripheral blood binuclear lymphocytes was applied. Per each sample, 1000 binuclear lymphocytes were analyzed and total number of MN, number of cells with MN and their distribution (number in cells) determined. Micronuclei were determined in accordance with recommended criteria of HUMN (Human Micro Nucleus) Project (Fenech et al., 2003). Conventional Moorhead method was used on short-term cultures for 48 hr, with all cells being in the first division (Moorhead et al., 1960). Slides from each culture were numbered and anonymously scored. At least 200 well-spread metaphases with 46 ± 1 centromeres were examined. CAs were further subclassified as CSAs (including chromosome-type breaks, ring chromosomes, marker chromosomes, and dicentrics) and chromatid-type aberrations (CTAs; including chromatid-type breaks). Gaps were not scored as aberrations.

Statistics

All variables were expressed as medians and interquartile ranges for continuous data with or without normal distribution, respectively. Nonparametric data were compared between groups using the independent samples Mann–Whitney U-test. Additionally Spearman's correlation was used as measure of association

for continues variables. P-value <0.05 was considered statistically significant. All statistical analyses were performed using the computer software Statistical Package for the Social Sciences, version 20.0 (SPSS, Chicago, IL).

Results and discussion

The study population comprised of 198 participants, 98 males and 100 females divided in 5 age groups (20-29, 30-39, 40-49, 50-59, and 60-69). In the total group MN median is determined to be (0.00-2.00), average 1.05±0.07, while individual values ranged from 0 to 8 MN per 1000 binuclear cells. Median for cells with one MN (1 MN) was 2.00, while for two MN (2 MN) was 0.00.

In 21 female and 29 male samples no MN were found. Out of total female samples (n=100), cells with 1 MN were found in 79 of them. While in the male group (n=98), cells with 1 MN were found in 69 samples (Table 1). Cells with 2 MN were found in 25/100 female samples, and in only 12/98 male participants (Table 2). Correlation between gender and MN, for cells with 1 MN showed rho=0.135 p=0.059, while the values for cells with 2 MN were rho=0.170 p=0.017. Thus, the correlation between gender and 2 MN is stronger.

Relation between age and number of cells with MN is statistically significant p<0.000. For each additional year of age the risk of having 1MN (odds ratio) increases by additional 12%, 95% CI (8-16%). For each additional year of age the risk of having 2 MN increases by 6%, 95% CI (3%-10%). Risk for 2 MN is 3 x higher in females, 95% CI (from 1 to 6 times).

As shown in Figure 1 increased frequency of CAs correlates with age in both genders, whereas in females the peak of CAs occurrence is around the age of 50, which may be linked to with the changes in hormonal status and adjustment for menopause. In males, CAs frequencies peaked at the age of 60 and all were statistically significant according to Spearman's correlation (p<0.01; rho=0.463; rho=0.470; rho=0.455; respectively). Significant positive correlation between the age and number of aberrations within all groups in healthy population is evident. (rho=0.462; p<0.01)

Mann-Whitney U test was used to determine the significance of CA frequency between male and female groups. Number of aberrations in female group is 1.0 (0.0-2.0) is higher than number of aberrations in male group 0.0 (0.0 – 2.0). This difference is statistically significant (p=0.046) (Figure 2).

Table 1. Analysis of the influence of gender status on frequencies of cells with 1 MN

			Cells with 1 MN								Total
			.00	1.00	2.00	3.00	4.00	5.00	6.00	8.00	
gender	male	Number	29	23	22	15	6	3	0	0	98
		%	29.6%	23.5%	22.4%	15.3%	6.1%	3.1%	.0%	.0%	100.0%
	female	Number	21	23	22	15	9	5	4	1	100
		%	21.0%	23.0%	22.0%	15.0%	9.0%	5.0%	4.0%	1.0%	100.0%
Total		Number	50	46	44	30	15	8	4	1	198
		%	25.3%	23.2%	22.2%	15.2%	7.6%	4.0%	2.0%	.5%	100.0%

Table 2. Analysis of the influence of gender status on frequencies of cells with 2 MN

			Cells with 2 MN				Total
			.00	1.00	2.00	3.00	
gender	male	Number	86	10	1	1	98
		%	87.8%	10.2%	1.0%	1.0%	100.0%
	female	Number	75	16	7	2	100
		%	75.0%	16.0%	7.0%	2.0%	100.0%
Total		Number	161	26	8	3	198
		%	81.3%	13.1%	4.0%	1.5%	100.0%

We have found significant effect of gender – female had increased number of CAs – chromatid type, and of the age in both genders.

Micronuclei in healthy population lymphocytes are indicators of genome damage in single cell accumulated over the years, and the mutations that appear in the first in vitro cell division (Kopjar et al., 2010). Today, cytogenetics provides numerous biomarkers for evaluating chromosomal instability and scoring of MN in lymphocytes is one that draws a lot of interest (Milošević-Djordjević et al., 2012). In this study we have analyzed the level of cytogenetic damage in healthy population from Bosnia and Herzegovina, addressing both genders and within the range of age approaching of professionally exposed and actively working population. That population could be controlled using MN-test and CA, in the future. Contrary to the published studies, in our research we did not count the total number of micronuclei (1+2 MN) they were rather been given as number of cells with 1 MN and number of cells with 2 MN. Considering that the subject group included healthy individuals with no medical record of acute infections or medical exposures or records of exposure to chemical and/or physical agents in the months preceding the study the influence of large number of environmental factors on single MN test values were minimized. Therefore gender and age were considered factors with largest influence on MN formaiton (Fenech and Bonassi, 2010; Battershill et al., 2008).

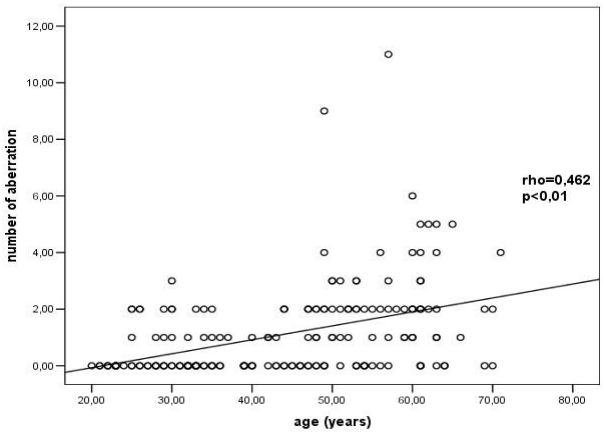

Figure 1. Relation among age and number of aberrations at all groups.

Our research, as well as others presented, show that females have higher MN count than males, on average. According to Fenech (Fenech, 1998) the frequency of MN in females is 1.2 to 1.6 times higher. Our research shows that females may have up to 3 times more cells with 2 MN than males, while this ratio is smaller when 1 MN is considered. One of the reasons

why females do have increased frequency of MN is the loss of one of X chromosomes through MN (Bolognesi et al., 2006; Kažimirova et al., 2006).

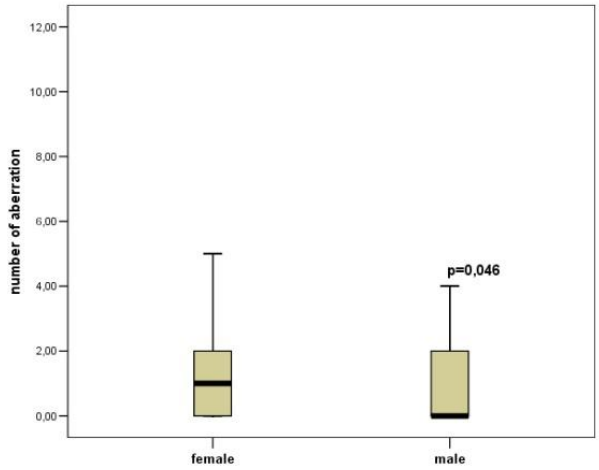

Figure 1. Relation among number of aberrations at both genders.

Positive correlation among MN frequency and age was reported in numerous studies (Veerachari et al., 2011; Nefic and Handzic, 2013; Jones et al., 2012). According to our data, age had statistically significant influence on the counts of cells with both 1 and 2 MN. The research showed that each additional year of age adds additional 6% to the risk of increase of number of cells with 2 MN, 95% CI, while that percentage is 12%, even higher when considering cells with 1 MN. Milosevic-Djordjevic et al. (2012) showed a decrease of MN frequencies in groups of older age and explained that this might be the result of declining in proliferation capacity of cells with aging. Norppa and Falck (2003) upon extensive analysis of numerous studies results, concluded that 30% to 80% of spontaneously arisen MN comprised whole chromosomes. In contrast to sex chromosomes that are more often lost through MN, autosomes appear randomly in MN and cannot be lost by ageing only (Norppa and Falck, 2003; Bukvic et al., 2001). It has also been noted that apparently healthy individuals may have large number of MN containing specific chromosome (Kopjar et al., 2010). We have found significant effect of gender – female had increased number of CAs – chromatid type, and of the age in both genders. Our results concur with other investigations done recently in other European countries, though we were not able to correlate CAs frequency and cancer risk due to inability to establish long-term follow-up. Frequency distribution of CTAs and CSAs between male and female showed predominance of CTAs over CSAs, independently of gender. An increase in chromosomal aberrations may be due to either genetic or acquired conditions conferring higher susceptibility to genetic damage. Elevated levels of chromosomal aberrations in peripheral blood lymphocytes may be seen as an indicator of an early phase of carcinogenesis, where various genetic alterations are also generated in different tissues (Mitelman et al., 2004).

Upon present experience and knowledge, MN test has great advantage over other cytogenetic tests since it allows damage estimation at functional level and integration of mitotic spindle, which other methods cannot achieve (Bolognesi et al., 2006). It should be pointed out that MN-test is useful in genome instability discovery that is in relation with increased cancer risk (Fenech and Crott, 2002). According to Bonassi research, this study provides preliminary evidence that MN frequency in peripheral blood lymphocytes is predictive of cancer risk, suggesting that increased MN formation is associated with early events in carcinogenesis (Bonassi et al., 2011).

Conclusions

The results of MN test and CA analyzed over age and gender obtained in this study are in accordance with the results determined on general healthy population in other research. These results will facilitate construction of research database for multiple genetic

categorization and for comparing and interpreting analysis in further research projects, medical diagnostics, control and bio-monitoring for professionally and environmentally exposed populations and others. That is the applicable character of this research. Also, the results are the ground for further development of laboratory database.

Acknowledgment

This research was funded by Federal Ministry of education and science in 2010, Federation of Bosnia and Herzegovina. It should be noted that all the participants signed written informed consent prior to the participation in the study.

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COMPARISON OF TWO DIFFERENT MULTIPLEX SYSTEMS IN CALCULATING KINSHIP AMONG CLOSE RELATIVES

Mirjana Beribaka[1*], Selma Hafizović[2], Amela Pilav[3], Mirela Džehverović[3], Damir Marjanović[2,4,5], Jasmina Čakar[3]

[1]*University of East Sarajevo, Faculty of Technology, Zvornik, Bosnia and Herzegovina*
[2]*University of Sarajevo, Faculty of Science, Sarajevo, Bosnia and Herzegovina*
[3]*Institute for Genetic Engineering and Biotechnology, Sarajevo, Bosnia and Herzegovina*
[4]*International Burch University (IBU), Sarajevo, Bosnia and Herzegovina*
[5]*Institute for Anthropological Research, Zagreb, Croatia*

***Correspondence**
E-mail: mirjana.beribaka@gmail.com

Received
October, 2016
Accepted
February, 2017
Published
June, 2017

Research article

Abstract

This study compares the results obtained using two multiplex systems, PowerPlex® 16 System and PowerPlex® Fusion System, to evaluate the probability of a specific kinship relationship between the offspring of three pairs of identical twins, such as full kinship (siblings), first-degree relatives (first cousins) and half-siblings. Genomic DNA was isolated and amplified from buccal swab and selected short tandem repeat (STR) markers were detected. Electropherograms were generated and analyzed for all persons, using two multiplex systems. Paternity testing for every nine offspring of six examined couples was performed and in all cases the probability that the alleged father is the true father, was over 99.9999%. Kinship analyses were performed setting up two different hypotheses and calculating the likelihood ratio (LR) and kinship probability. Determining the degree of kinship between persons who were full siblings, likelihood ratio showed the highest values contrary to other two types of kinship. Kinship analyses between first cousins showed a higher probability that the examined persons are half-siblings, rather than they are first cousins. In most cases, the introduction of additional seven loci included in PowerPlex® Fusion System increased the values of average likelihood ratios. It is recommendable to use over 20 STR loci in complex kinship analyses.

Key words: *kinship, identical twins, PowerPlex® 16 System, PowerPlex® Fusion System, likelihood ratio*

Introduction

Personal identification or complex kinship analyses represents the most challenging tasks for forensic investigators. Determining sibship or half sibship among close relatives is a frequent issue in kinship cases. These analyses can be carried out by testing a set of STR (Short Tandem Repeat) loci. STR allelic variants vary from person to person, so it is not unusual that two persons share the same alleles at STR loci, or even to match at two or three STR loci. However, the minimum theoretical probability

that two individuals share identical alleles at all 15 STR loci included in the commercial multiplex STR system PowerPlex® 16 for the Caucasian population is 1/1.83 x 10^{17} (Marjanovic and Primorac, 2013).

STR markers became very popular in forensic practice because they are based on PCR technology, and they can be used when it comes to a small amount, or degraded DNA. STR markers are suitable for multiplex amplification and include sensitive fluorescent detection, which allows researchers to quickly collect the data based on the markers. STR markers have a great power of discrimination between persons who are not related, but also between closely related individuals (Butler, 2015).

A large number of multiplex STR systems have been developed and they are used in forensic practice for many purposes, like confirming or excluding paternity, but also, for other types of relationships between individuals. For example, Thomson et al. (2001) analyzed the kinship relations using multiplex STR loci and Gaytmenn et al. (2002) studied the sensitivity and specificity of kinship analysis. Reid et al. (2004) compared the probability of sibship for pairs of full siblings versus unrelated individuals, using Identifiler multiplex STR kit (15 STR loci plus amelogenin). In this study, LR values for known full siblings ranged from 4.6 to over 1 billion and for unrelated individuals from 0.000000045 to 0.12. All full siblings had an LR > 1 and all nonsiblings had an LR < 1.

PowerPlex® 16 System allows co-amplification and three-color detection of sixteen loci (fifteen STR loci and Amelogenin) including Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818 (Promega Corporation, 2013). The PowerPlex® Fusion System is a 24-locus multiplex for human identification applications and allows co-amplification and fluorescent detection of the 13 core CODIS loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11), the 12 core European Standard Set loci (TH01, vWA, FGA, D21S11, D3S1358, D8S1179, D18S51, D10S1248, D22S1045, D2S441, D1S1656 and D12S391) and Amelogenin for gender determination. This system also includes DYS391, as a male specific locus. Penta D and Penta E loci are included in order to increase the discriminatory power and enable searching the databases that contain profiles with these loci. Finally, D2S1338 and D19S433 loci, which are contained within large number of databases, are incorporated to further increase the power of discrimination (Promega Corporation, 2014).

Appropriate application of statistical model plays an important role in the determination of kinship between individuals. To calculate the kinship between two individuals, it is necessary to define the relatedness coefficients for each type of relations. The relatedness coefficients are commonly referred as k_0, $2k_1$, k_2 (for two observed persons, X and Y): k_0 = P (none of allele X is identical by descent to Y alleles); $2k_1$ = P (one allele X is identical by descent to one of the Y allele, but the second one is not); k_2 = P (both X alleles are identical by descent to Y alleles), where P is the probability for each individually analyzed locus (Fung and Hu, 2008). The probability of kinship is calculated based on these coefficients.

The aim of this study was to compare the performance of two multiplex systems, PowerPlex® 16 and PowerPlex® Fusion, in the process of statistical determination of kinship between the descendants of three pairs of identical twins. The specificity of this study is that the descendants of identical twins are first cousins, but regarding their DNA profiles, they are half siblings. The main objective was to

investigate whether larger number of loci contained within PowerPlex® Fusion System, increases the possibility of discrimination between three types of kinship: full sibship, first cousins and half sibship.

Materials and methods

Samples were collected from 21 individuals, of which three pairs of identical twins with their spouses and with a total of nine offspring (Fig. 1). The first twin pair is labeled as D and N, their spouses are A and T, and their children are DA1, DA2, NT1 and NT2. The second twin pair is labeled as M and B, their spouses as V and T1, and children as MV, BT1 and BT2. The third twin pair is labeled as AD and AD1, their spouses as SA and ME and their children as ASA and AMS. All persons investigated gave informed consent.

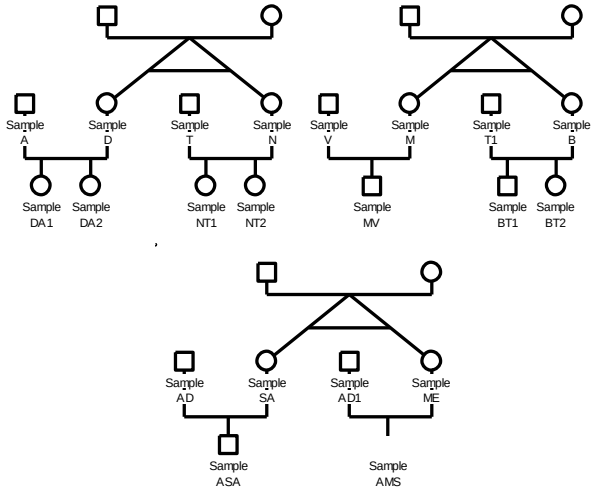


Figure 1. Pedigrees for three pairs of identical twins

DNA was isolated and amplified from buccal swab of all persons, according to the modified Miller's protocol (Miller, Dykes and Polesky, 1987) and selected STR markers were detected using ABI PRISM 310 Genetic Analyzer and two multiplex systems.

Data analysis was performed using GeneMapper® ID software, ver. 3.2. Paternity testing for all the descendants of six examined couples was performed. Kinship analyses were performed by setting up two different hypotheses and calculating the LR. Calculations were performed using EasyDNA_2Persons software, suggested by Fung and Hu (2008). Before starting the software analysis, it was necessary to make one input file, which contains a list of all 15 loci, allelic variants and their frequencies for PowerPlex® 16 System, or 22 for PowerPlex® Fusion System. This software uses relatedness coefficients to describe the type of relationship between two persons under the hypothesis (Tab. 1).

Table 1. Relatedness coefficients (k_0, $2k_1$, k_2) for some common relationships between two persons (Fung and Hu, 2008)

Relationship	k_0	$2k_1$	k_2
Parent-child	0	1	0
Full siblings	1/4	1/2	1/4
Half siblings	1/2	1/2	0
Grandparent-grandchild	1/2	1/2	0
Uncle-nephew	1/2	1/2	0
First cousins	3/4	1/4	0
Second cousins	15/16	1/16	0

Hypothesis testing and calculation of LR was performed for each locus individually, using the formulas for the calculation of probability, as shown in Table 2.

Table 2. Likelihood ratio from with two opposing hypotheses Hp: (Y, Z) ~ (x0, 2x1, k2) versus Hd: (Y, Z) ~ (1, 0, 0) (Fung & Hu, 2008)

Y	Z	Likelihood ratio
A_iA_i	A_iA_i	$k_0 + 2k_1/p_i + k_2/p_i^2$
A_iA_i	A_iA_j	$k_0 + k_1/p_i$
A_iA_i	A_jA_j	k_0
A_iA_i	A_jA_k	k_0
A_iA_j	A_iA_j	$k_0 + k_1(p_i+p_j)/(2p_ip_j) + k_2/(2p_ip_j)$
A_iA_j	A_iA_k	$k_0 + k_1/(2p_i)$
A_iA_j	A_kA_l	k_0

As an input file for EasyDNA_2Persons software, we used the list of loci and allelic variants for the Bosnian-Herzegovinian population (Marjanovic et al., 2006). Profiles, obtained using PowerPlex® Fusion System, include seven additional loci, whose allelic frequencies were taken from the data for Croatian population (Curic, Gasic, Pluzaric and Smiljcic, 2012).

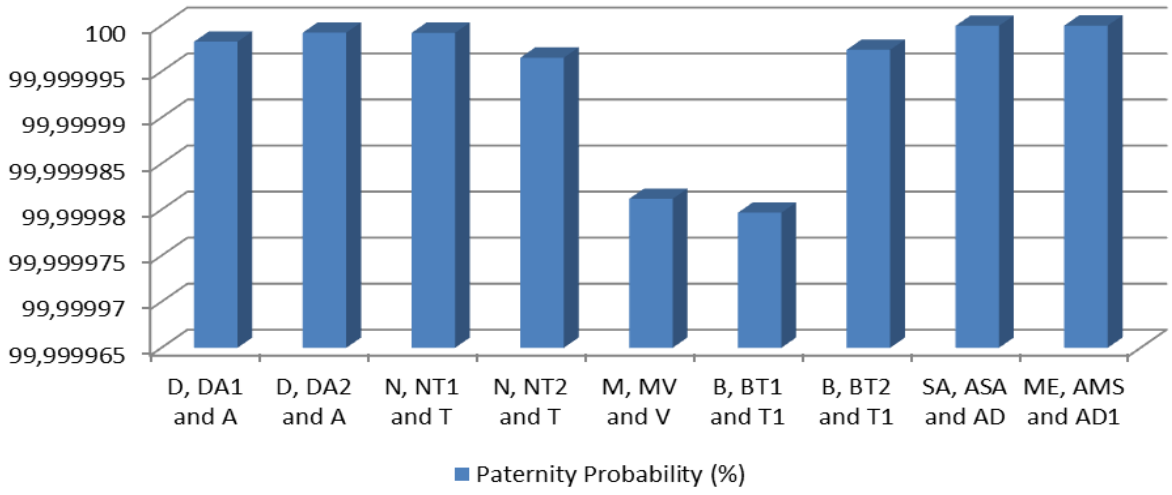
Results and discussion

Electropherograms were generated and analyzed using two multiplex systems, where both multiplex systems gave satisfactory results for all individuals. DNA samples were profiled using PowerPlex® 16 System and PowerPlex® Fusion System. Samples were analyzed using the ABI PRISM 310 Genetic Analyzer and the profiles were obtained using GeneMapper® ID software, ver. 3.2.

In all the cases of paternity testing using the PowerPlex® 16 System, probability that the alleged father is the true father, was higher than 99.9999%. There were some variations in the values of CPI in some samples, which reflected on the probability of paternity. The chart shows that the lowest probability of paternity had parental couple labeled as M and V, for the child labeled as sample MV (99.99998%) and a parental couple labeled as B and T1, for the child labeled as sample BT1 (99.999975%) (Fig. 2).

Other samples had similar values, except for the pair of samples AD and AD1, where in both cases of paternity testing were obtained much higher probabilities, compared to the other tested samples. High CPI values resulted from rare allelic variants present at several loci. In this case, Penta E locus was especially interesting, since twin pairs with the highest probability of paternity, had allelic variant 20. According to Marjanovic et al. (2006), this allelic variant was not observed in a sample of 100 individuals in the Bosnian-Herzegovinian population, so allelic frequency of variant 20 is considered as 0.005. Another allelic variant that was not observed by Marjanovic et al. (2006) is 14.3 at the D1S1656 locus, which was found in the sample T.

Having confirmed the paternity for every descendant of all twin pairs, calculation of the degree of kinship could be performed. The examined types of kinship were: full sibship, first-degree relatives and half sibship. It



was

Figure 2. Paternity testing for all samples using the PowerPlex® 16 System

Table 3. Analysis of the relationships of full siblings versus first cousins, for all descendants

	PowerPlex® 16 System		**PowerPlex® Fusion System**	
	Average LR	Kinship Probability (%)	Average LR	Kinship Probability (%)
Samples DA1 and DA2	2.9458	74.66%	10.1532	91.03%
Samples DA1 and NT1	0.5106	33.80%	0.9545	48.84%
Samples DA1 and NT2	1.234	55.24%	0.2656	20.99%
Samples DA2 and NT1	0.1114	10.02%	0.0021	0.2%
Samples DA2 and NT2	0.0789	7.31%	0.0058	0.58%
Samples NT1 and NT2	921.7813	99.89%	34707.44	99.99%
Samples BT1 and BT2	2399.483	99.96%	499822.1	99.99%
Samples MV and BT1	0.748	43.95%	0.1962	16.4%
Samples MV and BT2	0.0377	3.36%	0.0333	3.22%
Samples ASA and AMS	0.0337	3.26%	0.0054	0.54%

necessary to set up two hypotheses (Hp and Hd) and to calculate LR or probability factor. If the value of LR is greater than 1, it supports the probability of Hp hypothesis, and if the value is less than 1, it supports probability of Hd hypothesis.

Firstly, we tested the following two hypotheses: Hp - The relationship between the examined persons is full kinship (brothers and sisters) and Hd - The relationship between the examined persons is first-degree relatives (first cousins). The obtained average LRs for both multiplex systems are presented in Table 3.

Average LRs for fully related samples using PowerPlex® 16 were higher than 1, but varied in their values. LR for DA1 and DA2 samples was 2.9458, for NT1 and NT2 samples was 921.7813 and for BT1 and BT2 samples was 2399.483. These differences between LRs resulted from the fact that some samples have a high number of loci where they share the same alleles, whether they are homozygote or heterozygote, or they share some rare alleles with low allelic frequencies. For the same reason, samples DA1 and NT2 have an average LR over 1 (1.234). This result could indicate that there is a probability that samples DA1 and NT2 are full siblings, even though they are first cousins. The use of 22 loci in PowerPlex® Fusion System gave better confirmation of supposed relations in 9 out of 10 sample pairs. Additional loci solved the dilemma about the samples DA1 and NT2, where the average LR decreased under 1 (0.2656), so hypothesis Hp could be rejected and the samples DA1 and NT2 could be considered as not full siblings. Distinguishing between full siblings and first degree relatives requires larger number of STR loci than 15. The PowerPlex® Fusion profiles also confirmed the full kinship relations between samples DA1 and DA2, NT1 and NT2 and BT1 and BT2, but with much greater reliability (10.1532, 34707.44 and 499822.1, respectively). PowerPlex® Fusion System contains two loci (D2S1338 and D12S391) that are recommended by Yuan et al. (2017) as loci with higher discrimination power. STRs with higher discrimination power values should be included in analyses when additional autosomal markers are required for full sibship identification. Next, we tested the following two hypotheses: Hp - The relationship between the examined persons is full kinship (brothers and sisters) and Hd - The relationship between the examined samples is half siblings (half-brothers and half-sisters). Results are presented in Table 4. Regarding the probabilities of full sibship

Table 4. Analysis of the relationships of full siblings versus half siblings, for all descendants

	PowerPlex® 16 System		**PowerPlex® Fusion System**	
	Average LR	Kinship Probability (%)	Average LR	Kinship Probability (%)
Samples DA1 and DA2	1.0358	50.88%	1.9523	66.13%
Samples DA1 and NT1	0.1258	11.17%	0.0647	6.08%
Samples DA1 and NT2	0.4324	30.19%	0.1055	9.54%
Samples DA2 and NT1	0.0579	5.47%	0.0027	0.27%
Samples DA2 and NT2	0.0487	4.64%	0.004	0.4%
Samples NT1 and NT2	43.376	97.75%	307.191	99.67%
Samples BT1 and BT2	188.626	99.47%	4963.224	99.98%
Samples MV and BT1	0.3554	26.22%	0.0534	5.07%
Samples MV and BT2	0.0130	1.28%	0.0033	0.33%
Samples ASA and AMS	0.039	3.75%	0.0043	0.43%

versus half sibship, average LRs in all samples decreased (Tab. 4), comparing to analysis of full sibship versus full cousins (Tab 3). This is the result of relatedness coefficients being lower for first degree relatives, than for half sibling. It was also demonstrated in the study of Von Wumb-Schwark et al. (2015) that LR decreased in full related individuals after analyzing full sibship versus half sibship, comparing to the full sibship versus unrelated persons.

All cases of full sibship were again confirmed, in both multiplex systems. It is also noticable that in all analyses, additional loci in the PowerPlex® Fusion System gave higher support to supposed relations, ie. higher values for full sibship in fully related individuals and lower values of full sibship in half related individuals.

Studies suggest that genotyping more than 20 autosomal STR loci improve forensic personal identification, especially in the sibship analyses (Allen et al., 2007; Carboni et al., 2014; Von Wumb-Schwark et al., 2015; Tamura et al., 2015; Turrina et al., 2016), which corresponds to our findings.

Finally, two hypotheses were tested: Hp - The relationship between the examined samples is half siblings (half-brothers and half-sisters) and Hd - The relationship between the examined persons is first-degree relatives (first cousins). The fully related individuals were excluded from this analysis (Tab. 5). All results obtained with the PowerPlex® 16 System gave average LR values

Table 5. Analysis of the relationships of half siblings versus first cousins, for all descendants

	PowerPlex® 16 System		**PowerPlex® Fusion System**	
	Average LR	Kinship Probability (%)	Average LR	Kinship Probability (%)
Samples DA1 and NT1	4.0609	80.24%	10.2145	91.08%
Samples DA1 and NT2	2.8555	74.06%	2.5191	71.58%
Samples DA2 and NT1	1.9246	65.81%	0.7757	43.68%
Samples DA2 and NT2	1.6202	61.83%	1.4643	59.42%
Samples MV and BT1	2.1098	67.84%	3.6839	78.65%
Samples MV and BT2	2.8924	74.31%	10.0634	90.96%
Samples ASA and AMS	1.3949	58.24%	2.0016	66.68%

higher than 1, indicating the confirmation of half sibship. Considering the fact that identical twins share the same DNA profile, their descendants can be perceived as half siblings. The introduction of additional seven loci used in the PowerPlex® Fusion System increased the values of LR in 5 out of 7 analyzed pairs. Carboni et al. (2014) reported a case study of two women who were cousins and they suspected to be daughters of the same father. Their mothers were sisters and were not available for kinship testing. The analysis with a commercial kit showed a probability value of about 15, to support the hypothesis that they were half-sisters. Including the additional 26Plex markers, the total of 41 loci, probability value increased to 435, supporting the hypothesis that the subjects were half-sisters. Our results also suggest the use of larger number of loci when distinguishing between full and half sibship.

Conclusions

In this study, we compared two multiplex systems, PowerPlex® 16 System and PowerPlex® Fusion System, regarding the determination of kinship between the descendants of identical twins. Likelihood ratios obtained by calculating the degree of kinship between first cousins provided greater support to the hypothesis that they are half siblings. In all cases of full siblings, likelihood ratio the most strongly supported the hypothesis of full sibship.

Additional seven loci in PowerPlex® Fusion System gave more accurate results, so it is recommendable to use 20 or more STR loci in analysis of different types of kinships to avoid ambiguous results and to facilitate interpretation of obtained results.

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AIR POLLUTION TOLERANCE INDEX OF *Plantago major* IN STEELWORKS AREA OF ZENICA, BOSNIA AND HERZEGOVINA

Tajna Klisura[1], Adisa Parić[1], Mirel Subašić[2], Erna Karalija[1*]

[1]*University of Sarajevo, Faculty of Science, Sarajevo, Bosnia and Herzegovina*
[2]*University of Sarajevo, Faculty of Forestry, Sarajevo, Bosnia and Herzegovina*

***Correspondence**
E-mail: erna.karalija@gmail.com

Received
November, 2016
Accepted
May, 2017
Published
June, 2017

Research article

Abstract

Atmospheric pollution is among the largest anthropogenic impacts on the ecosystem. In numerous studies it was observed that plants, especially those that grow in urban areas, are heavily influenced by different pollutants and their survival is correlated with structural and metabolic adaptation to stressful environmental conditions. Primary objective of this study was to determine the index of tolerance to air pollution (APTI) of plantain (Plantago major), on two locations in Zenica. The results indicated that index of tolerance to air pollution of P. major, APTI, is higher in individuals sampled from the contaminated site, than those in the control area.

Key words: *air pollution, plantain, APTI*

Introduction

Plants take an active part in the circulation of nutrients and gases such as carbon dioxide, oxygen and also provide an enormous leaf surface for the absorption and accumulation of air pollutants resulting in reduction of the pollution level in the environment (Escobedo et al., 2008). Atmospheric pollution is among the largest anthropogenic impacts on the ecosystem (Hijano et al., 2005). In numerous studies it was observed that the plants, especially those that grow in urban areas, are heavily influenced by different pollutants and their survival is correlated with structural and metabolic adaptation to stressful environmental conditions (Gostin, 2009). Sensitivity and responses of plants to air pollution are very variable. Plant species that are more sensitive are used as biological indicators of air pollution. Responses of the plants to air pollution on physiological and biochemical levels can be understood by analysing the specific factors that determine the resistance and sensitivity. Urban areas can be contaminated by many pollutants such as SO2, CO, NO and heavy metals and the plants that grow there are exposed not just to one pollutant but a variety of pollutants including their complex interactions (Agarwal, 1985; Tiwari et

al., 1993). The main objective of this study was to determine the air pollution tolerance index (APTI) of *Plantago major*, collected from two sites in the Zenica area in order to determine the tolerance of this species to the existing atmospheric conditions.

Materials and methods

Study area

Sampling of plant material was performed in October 2015 in two localities in the area of Zenica: Tetovo and Smetovi. Tetovo is located at latitude 44°13'51.18" and longitude 17°53'19.71" and Smetovi at latitude 44° 14'41.77" and longitude 17° 58'42.05". Tetovo is located near steelworks ArcelorMittal, the source of air pollution. Plants collected at Smetovi locality were used as control samples.

The samples were collected in three biological replicates (fully developed leaves of *P. major*) and transferred into the Laboratory of Plant Physiology, Faculty of Science, University of Sarajevo. The samples were stored in a refrigerator at +4°C for the purpose of further analysis.

Relative Water Content (RWC)

Relative water content was analysed according to Liu and Ding (2008). Five leaf samples of each individual per investigated site were selected for analysis. Fresh weight was obtained by weighing the fresh material. The leaves were then immersed in water overnight, for the purpose of determination of turgid mass. The leaves were than dried in an oven at 70°C overnight and reweighed to obtain the dry weight. After determining the parameters of fresh, dry and turgid weight of the leaves, relative water content was calculated using the formula:

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} \times 100$$

where FW is fresh weight, DW is dry weight and TW is turgid weight.

Leaf extract pH

Leaf extract pH was determined according to Singh and Rao (1983). Fresh plant material was homogenized in a mortar with the addition of 10 ml distilled water. The content of the mortar was then filtered and pH of the leaf extract determined using pH meter (872 pHlab Metrohm, Swissmade) after calibration with buffer solutions at pH 4 and pH 7.

Total chlorophyll and carotenoid content

Extraction of pigments was carried out from fresh plant material (0.25 g), by maceration in 80% acetone (v/v). Following the centrifugation of the macerate (Biofuge A centrifuge manufacturer Heraeus sepatech) at 1000 rpm, for 15 minutes, the supernatant was collected for further analysis.

Quantities of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were determined by absorbance measurement at 663 nm (chlorophyll a), 646 nm (chlorophyll b) and 440 nm (carotenoids) using spectrophotometer (*Perkin-Elmer spectrophotometer Lambda 25*). Calculation of chlorophyll a and chlorophyll b and carotenoid contents was done according to the formulas by Porra et al. (1989) and Holm (1954):

Chl a = 12.25A663 - 2.55A646 (µg/g)

Chl b = 20.31A646 - 4.91A663 (µg/g)

Chl a + b = 17.76A646 + 7.34A663 (µg/g)

Car= 4.69A440 - 0.267Chl a + b (µg/g)

Ascorbic acid (AA)

Extraction of ascorbic acid was carried out from 0.5 g of fresh material, in 5 ml of distilled water and 2-3 drops of glacial acetic acid (99.8%), obtained supernatant was used for analyses.

Titrimetric analysis of ascorbic acid was carried out according to the following procedure: a solution of 10 ml of the filtrate, 50 ml of distilled water and 0.5 ml of 1% starch was mixed and immediately titrated to endpoint with a standardized solution of iodine (0.002 mol L-1). Titration was repeated twice for samples from both sites. Results are expressed in mg/g (Tahirović et al., 2012).

Air Pollution Tolerance Index (APTI)

Air Pollution Tolerance Index (APTI) was determined by Singh and Rao (1983) using the formula:

$$APTI = \frac{A\,(T+P)+R}{10}$$

where A is ascorbic acid content (mg/g FW), T is total chlorophyll content (mg/g FW), P is pH of leaf extracts and R is relative water content (% of the leaves).

Results and discussion

Relative Water Content

To calculate relative water content in *Plantago major*, values of fresh, turgid and dry mass of leaves were determined. The results (Table 1) indicate that individuals sampled at the site of Tetovo, show increase in relative water content in relation to the locality Smetovi, control site. Dhanam et al. (2014) obtained similar results by recording higher relative water content in leaves from polluted sites than in those at the control site.

These results suggest that relative water content plays an important role in maintaining homeostasis in plants that are under stress due to air pollution. Plant species with high relative water content are tolerant to pollutants. High relative water content in plant allows it to maintain physiological homeostasis when exposed to pollutants when transpiration rates are usually high. It also serves as an indicator of plant resistance to drought conditions (Dhanam et al., 2014).

Leaf extract pH

Leaf extract pH is another necessary parameter in determination of APTI. Variation was observed in pH values of leaf extracts depending on sampling location. The pH of the extract of leaves (Table 1) sampled from the contaminated site (5.85) was lower than the values recorded at control sites (8.6).

Leaf extract pH has a very important role in determining the level of tolerance of plants to any kind of pollution. High pH can affect the efficiency of conversion of hexoses into ascorbic acid, while at low pH revealed good correlation with sensitivity to air pollution and with reduced photosynthetic activity in plants. Higher pH allows greater tolerance of plants to air pollution (Dhanam et al., 2014).

Photosynthetic activity was reported to be very dependent upon the pH of the leaf (Yan-ju and Hui, 2008) and photosynthesis is reduced in plants with low leaf pH values (Turk and Wirth, 1975). In a study on the individuals from two different sites it was found that plants stationed in the industrial contaminated area had an acidic pH value, while individuals in the control area showed neutral to slightly basic pH (Rai et al., 2013), which is in line with the results obtained on P. major.

Content of photosynthetic pigments

Analysis of the content of photosynthetic pigments included the determination of the concentrations of chlorophyll a, chlorophyll b, total chlorophyll content and carotenoids. Analysis showed higher content of chlorophyll a, chlorophyll b and carotenoids in individuals sampled from the contaminated area (Table 1).

Chandawat et al. (2011) found that the chlorophyll content of all plant species tested varied in accordance with the level of pollution

Table 1. Air Pollution Tolerance Index (APTI) of *Plantago major* on two sites in Zenica

	Tetovo	**Smetovi**
Fresh weight (g)	0.72	0.47
Turgid weight (g)	0.82	0.54
Dry weight (g)	0.12	0.08
RWC (%)	85.32	82.96
Chlorophyll *a* (mg/g)	0.85	0.67
Chlorophyll *b* (mg/g)	0.22	0.21
Total chlorophyll (mg/g)	1.08	0.88
Carotenoids (mg/g)	0.28	0.25
pH	5.81	6.08
Ascorbic acid (mg/g)	1.23	0.70
APTI	9.37	8.78

in a given area, as well as in accordance with species tolerance and sensitivity to air pollution. Certain air pollutants reduce and others increase the content of total chlorophyll in the plant (Dhanam et al., 2014).

Tripathi and Gautam (2007) recorded large loss of chlorophyll in the leaves of plants that have been exposed to high levels of air pollution, which once again points to the role of chloroplasts in the plant and supports the argument that the chloroplast is a primary place of attack of air pollutants on the plant.

Ascorbic acid

The results (Table 1) show that the content of ascorbic acid is higher in individuals from contaminated sites (1.23 mg) compared to individuals in the control area (0.7 mg).

Ascorbic acid plays an important role in the synthesis of the cell wall in plants, photosynthetic carbon fixation and the cell division (Lakshmi et al., 2008). The increased value of ascorbic acid content may be caused by the defence mechanisms of the plant itself. The results obtained by Dhanam et al. (2014) concur with the research of Chandawat et al. (2011) and Rai et al. (2013) who observed higher levels of ascorbic acid in the leaves of the most tolerant plants and those plants stationed near polluted areas (Dhanam et al., 2014). Tripathi and Gautam (2007) noted large variations in the levels of ascorbic acid among all samples regardless of the collection site.

Elevated levels of ascorbic acid in plants that were located in the area with high levels of air pollution can be caused by higher rate of production of reactive oxygen species during photooxidation (Tripathi and Gautam, 2007). Elevated levels of ascorbic acid recorded in P. major from Tetovo locality could be attributed to plant defence mechanisms in conditions of high air pollution.

Air Pollution Tolerance Index (APTI)

APTI is calculated on the basis of biochemical parameters that were analysed in the study. All these parameters play very important role in determining the resistance and sensitivity of plant species. In this study, APTI values of plants from contaminated sites were higher when compared to the control (Table 1). Air pollution in urban and industrial areas can be

adsorbed, absorbed, accumulated or integrated into the plant, and the toxin can cause a variety of injuries to the plants. The level of injury will be high for sensitive and low for tolerant plant species. Sensitive species are indicators of air pollution while tolerant ones could be used for reducing levels of air pollution (Subrahmanyum et al., 1985). Species with APTI lower than 10 are characterized as sensitive to air pollution and can be used for biomonitoring levels of air pollutants (Agrawal et al., 1991). Tolerant plant species accumulate pollutants, so planting polluted areas with these plants has great benefit. Based on the data given in Table 1., it is evident that APTI values (9.37 at contaminated site; 8,78 at control site) place *P. major* among sensitive plant species that may be used as appropriate indicators of air pollution for studied area.

Conclusions

The results of four parameters studied within APTI support the hypothesis that *Plantago major* belongs to the species sensitive to air pollution, which is consistent with the classification of plants based on APTI (Singh and Rao, 1983). Air pollution is one of the greatest threats to the disruption of the ecological state of the environment. Due to the rise in industrialization, there is constant danger of deforestation caused by air pollution. These facts indicate the importance of determining Air Pollution Tolerance Index (APTI) for different plant species, as well as the use of obtained results in future planning and the establishment of control measures against air pollution.

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PREDICTION OF THE SIZE OF NANOPARTICLES AND MICROSPORE SURFACE AREA USING ARTIFICIAL NEURAL NETWORK

Dženana Sarajlić[1], Layla Abdel-Ilah[2], Adnan Fojnica[1*], Ahmed Osmanović[2*]

[1] *Graz University of Technology, Graz, Austria*
[2] *International Burch University, Sarajevo, Bosnia and Herzegovina*

*** Correspondence**
E-mail:
ahmed_osmanovic@hotmail.com
adnanfojnica@hotmail.com

Received
January, 2017
Accepted
May, 2017
Published
June, 2017

Abstract

This paper presents development of Artificial Neural Network (ANN) for prediction of the size of nanoparticles (NP) and microspore surface area (MSA). Developed neural network architecture has the following three inputs: the concentration of the biodegradable polymer in the organic phase, surfactant concentration in the aqueous phase and the homogenizing pressure. Two-layer feedforward network with a sigmoid transfer function in the hidden layer and a linear transfer function in the output layer is trained, using Levenberg-Marquardt training algorithm. For training of this network, as well as for subsequent validation, 36 samples were used. From 36 samples which were used for subsequent validation in this ANN, 80.5% of them had highest accuracy while 19.5% of output data had insignificant differences comparing to experimental values.

Key words: *artificial neural network, nanoparticles, polymeric nanoparticles, prediction, microspore surface area*

Research article

Introduction

A nanoparticle is the most fundamental component in the fabrication of a nanostructure, and is far smaller than the world of everyday objects that are described by Newton's laws of motion, but bigger than an atom or a simple molecule that are governed by quantum mechanics (Estelrich, 2014). In general, the size of a nanoparticle spans the range between 1 and 100 nm. Metallic nanoparticles have different physical and chemical properties from bulk metals (e.g., lower melting points, higher specific surface areas, specific optical properties, mechanical strengths, and specific magnetizations), properties that might prove attractive in various industrial applications. However, how a nanoparticle is viewed and is defined depends very much on the specific application (Horikoshi and Serpone, 2013). Polymeric nanoparticles (PNPs) are structures with diameter ranging from 10 to 100 nm. The PNPs are obtained from synthetic polymers,

such as poly-caprolactone, polyacrylamide and polyacrylate or natural polymers, e.g., albumin, DNA, chitosan gelatin (Hosseini et al., 2016). Based on in vivo behavior, PNPs may be classified as biodegradable, i.e., poly (L-lactide) (PLA), polyglycolide (PGA), and non-biodegradable, e.g., polyurethane. PNPs are usually coated with nonionic surfactants in order to reduce immunological interactions (e.g., opsonization or presentation PNPs to CD8 T-lymphocytes) as well as intermolecular interactions between the surface chemical groups of PNPs (e.g., van der Waals forces, hydrophobic interaction or hydrogen bond-ing). The application of biodegradable nanosystems in the development of nanomedicines is one of the most successful ideas (Wilczewska et al., 2012). Nanocarriers composed of biodegradable polymers undergo hydrolysis in the body, producing biodegradable metabolite monomers, such as lactic acid and glycolic acid (Pavot et al., 2014). Drug-biodegradable polymericnanocarrier conjugates used for drug delivery are stable in blood, non-toxic, and non-thrombogenic. They are also non-immunogenic as well as non-proinflammatory, and they neither activate neutrophils nor affect reticulo-endothelial system (Babahosseini, 2015). A few strategies can be employed for polymeric nanoparticles preparation: solvent evaporation, salting-out, dialysis, supercritical fluid technology, micro-emulsion, mini-emulsion, surfactant-free emulsion, and interfacial polymerization (Rao and Geckeler, 2011).

According to Rizkalla and Hildgen (2005), prediction of size of nanoparticles can be done using either Artificial Neural Network, Genetic alghoritm or Polynomial Regression Analysis. Based on their work, different batches were prepared by varying surfactant and polymer concentration as well as homogenization pressure. Two commercial ANN programs were used: Neuroshell predictor, a black-box software adopting both neural and genetic strategies and Neurosolutions, allowing a step-by-step building of the network. Results are then compared with those obtained by statistical method. It has been stated that Artificial Neural Networks offer a successful tool for nanoparticle preparation analysis and modeling.

Materials and methods

Obtaining desired nanoparticle's property, determination of their size, affinity and other important features is generally time, money and effort consuming (Giokas et al., 2010). Also, instruments used to carry out experiments are not widely available in any institute or research laboratories, so other more applicable tools are preferred to obtain results of interest. Artifical Neural Network (ANN) is one of those tools that are able to select data, create a network and evaluate its performance using mean square error (MSE) and regression analysis.

The network learns based on adjusting the interconnection weights between layers of input-output relations. Once the training is completed, ANN can predict outputs for new set of data when only input values are introduced. This describes the generalization ability of the network. Based on this, ANN system seems to be ideal for prediction of size and surface area determination (Singaram, 2011).

Feedforward, back-propagation, commonly used network in this type of research (Horikoshi and Serpone, 2013) is used for neural network training. The typical back-propagation network has an input layer- which consists of network inputs only. It is then followed by a hidden layer which consists of number of neurons, or hidden units which are placed in parallel. The network output is also formed by weighted summation that consists of outputs of the neurons in the hidden layer. Formed layer is called output layer. Usually number of output neurons equals

the number of outputs of the approximation problem. In this case, linear activation function for the output layer is used, since it is commonly used in regression problems, where powerful tool for predicting and interpreting information is needed (Wilczewska et al., 2012).

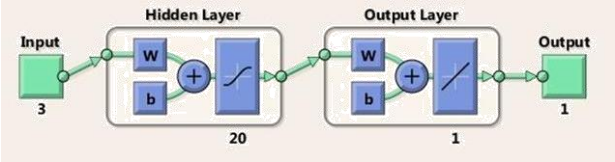

Figure 1. ANN architecture

TRAINLM is a network training function that updates weight and predicts values according to Levenberg-Marquardt optimization. Levenberg-Marquardt Algorithm (LMA) is commonly used training algorithm in data classification, as well algorithm that was used in previous experiment (Rizkalla and Hildgen, 2005). It is often the fastest backpropagation algorithm, although it does require more memory than other algorithms (Fojnica et al., 2016). Network performance function that is used is Mean Squared Error. It measures the network's performance according to the mean of squared errors. Mean Squared Error measures the network's performance according to the mean of squared errors. It is an average of the squares of differences between the actual observations and those predicted. The squaring of errors tends to heavily weight statistical outliers, affecting the accuracy of the results (Fojnica et al., 2016).

Input layer consisted of three network inputs that are followed by a hidden layer containing 20 neurons. Output layer consists of one neuron and output value parameter is the nanoparticle size (Figure 1). The network has been tested with different number of neurons in hidden layer so that best results for nanoparticle size could be observed. Thus, it was tested with 3, 7, 15, 20 and 31 neurons. Figure 1 illustrates ANN architecture and shows that with three parameters and 20 neurons in hidden layer ANN was trained to give final output-size of nanoparticles.

Input parameters to developed ANN were: concentration of biodegradable polymer in the organic phase, surfactant concentration in the aqueous phase and the homogenizing pressure. Output data was nanoparticle size which we have compared to experimental results in the research we were following (Figure 2). Absolute error was used for comparing the results obtained as outputs and NP size. Small absolute error means higher performance.

Absolute Error=|target(NP size)-ANN|

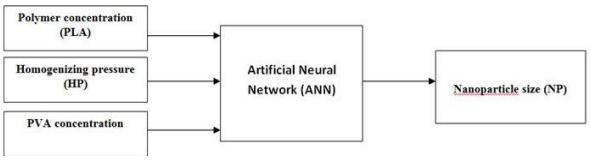

Figure 2. Block diagram of ANN for nanoparticle size

Data used from research paper (Hosseini et al., 2016), consisted of 36 samples implemented for training purposes. Also, they were used for the subsequent validation in order to determine the percentage of overlap with experimental data.

Results and discussion

In this study, impact of number of neurons in hidden layer, training functions as well as dataset distribution during training and transfer function on ANN output accuracy was examined. Based on the ANN performance, the optimal architecture was chosen for solving problem of prediction of the size of nanoparticles (NP) and microspore surface area (MSA). Five different networks were developed with different number of neurons in the hidden layers: 3, 7, 15, 20, and 31. The performance of networks (obtained from performance plot) was compared. As a result, network with 20 neurons achieved the best performance. Figure 3

represents performance plot of the network with 20 neurons in the hidden layer. It is evident that the training line perfectly follow its course however, validation and testing lines do not follow the training line and they almost overlap. Regardless, good results were obtained at the end of the process.

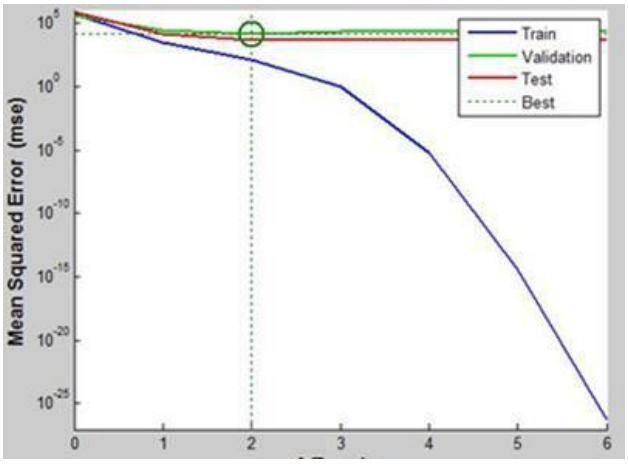

Figure 3. Performance plot of ANN

36 samples were used as a validation data. Absolute errors between outputs and targets were calculated and compared for each network. As a result, network with 20 neurons in the hidden layer has the lowest error rate, which means it has the best performance.

Table 1 (Appendix I) represents the results obtained from the validation process in terms of absolute error. It is clear that 22 out of 36 samples achieved 0% errors which is the best performance to be achieved. Errors appeared in 14 out of 36 samples.

Conclusions

In this study, Artificial Neural Networks (ANNs) were used to predict nanoparticle size and microspore surface area of polylactic acid nanoparticles, prepared by double emulsion method. Different batches were prepared while varying polymer and surfactant concentration, as well as homogenization pressure. In similar studies, researchers used two commercial ANNs programs: Neuroshell® Predictor, a black-box software adopting both neural and genetic strategies, and Neurosolutions®, allowing a step-by-step building of the network.

Since we did not have an opportunity to work with such commercial ANN programs, an ordinary ANN tool was used to predict nanoparticle size and microspore surface area of polylactic acid nanoparticles. So, in order to develop a system for prediction of nanoparticle size and microspore surface area, three variables were used as inputs: the concentration of the biodegradable polymer in the organic phase (5%, 7.5% and 10% w/v of PLA), surfactant concentration in the aqueous phase (0.1%, 0.5%, and 1% w/v of PVA) and the homogenizing pressure (5000, 10000, 15000 and 20000 psi of HP). NP size experimental data, in this case target data, were already given in the table of default research paper (Hosseini et al., 2016).

Neural network was trained with 36 samples and validated with 36 samples each with three different input variables. Training of Neural Network was performed on few occasions by using Matlab and its nnstart fitting tool. Every time the number of hidden neurons was changed and performances, regressions and Mean Square Errors (MSE) were recorded. So, at first ANN was trained with 3 and then with 7, 15, 20 and 31 neurons, until conclusion that the best results were recorded using 20 neurons. Thus, by selecting 20 neurons, the best possible performance and regression were reached and after inserting all samples in order to do subsequent validation, 22 out of 36 samples were obtained which completely match target data and show 0% relative error.

At the end, a comparison between two commercial ANN programs used in the research was made and we made the following conclusions:

1. Artificial neural networks offered successful tool for nanoparticle preparation analysis and modeling. Genetic algorithm represented fast and reliable method to determine the relative importance of inputs. Predictions from ANNs were closer to experimental values than those obtained using polynomial regression analysis.

2. Results obtained using NeuroSolutions® confirmed that a fexible, rather than a "black-box" program, was more advantageous as it would enable the free selection of different network parameters in manner appropriate for each problem. Also, pre-processing of the training data has proved to play an important role in modeling applications by neural computing.

Considering all things, ANNs represent a promising tool for the analysis of processes involving preparation of polymeric carriers and for prediction of their physical properties.

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Appendix 1

Table 1. Comparison of experimental target data with output data

Batch number	Polymer concentration (%w/v)	Pressure	PVA concentration (%w/v)	NP size (nm)	Outputs	Absolute error
1	5	5000	0.1	295.3	295.3	0%
2	5	5000	0.5	225.5	225.5	0%
3	5	5000	1	141.3	140.7	0.4%
4	5	10000	0.1	214.5	214.5	0%
5	5	10000	0.5	177.0	177.0	0%
6	5	10000	1	137.8	137.8	0%
7	5	15000	0.1	229.0	228.9	0.04%
8	5	15000	0.5	178.1	178.1	0%
9	5	15000	1	128.2	128.2	0%
10	5	20000	0.1	205.4	205.4	0%
11	5	20000	0.5	176.3	249.0	41.24%
12	5	20000	1	152.4	209.1	37.20%
13	7.5	5000	0.1	387.0	316.0	18.35%
14	7.5	5000	0.5	197.1	197.1	0%
15	7.5	5000	1	169.8	169.8	0%
16	7.5	10000	0.1	343.3	311.2	9.35%
17	7.5	10000	0.5	197.0	197.0	0%
18	7.5	10000	1	158.1	131.9	16.57%
19	7.5	15000	0.1	290.6	290.7	0.06%
20	7.5	15000	0.5	196.8	196.8	0%
21	7.5	15000	1	153.2	153.2	0%
22	7.5	20000	0.1	413.6	413.4	0.04%
23	7.5	20000	0.5	188.5	188.3	0.1%
24	7.5	20000	1	173.4	57.2	67.01%
25	10	5000	0.1	545.2	469.6	13.8%
26	10	5000	0.5	211.8	211.8	0%
27	10	5000	1	192.6	192.6	0%
28	10	10000	0.1	502.2	502.2	0%
29	10	10000	0.5	194.4	194.4	0%
30	10	10000	1	183.5	181.7	0.98%
31	10	15000	0.1	437.9	437.9	0%
32	10	15000	0.5	219.4	149.3	31,90%
33	10	15000	1	178.9	178.9	0%
34	10	20000	0.1	500.2	500.2	0%
35	10	20000	0.5	214.9	215.1	0.09%
36	10	20000	1	178.0	178.0	0%

From the Annals

The first known publication on populational genetics of B&H population.

Himmel H. (1887): Das Soldatenmaterial der Herzegowina in Antropologischer Beziehung. Mitteilungen der Anthropologischen Gesellschaft in Wien, Neue Folge VII. Band: 84-85.

Das Soldatenmaterial der Herzegowina in Antropologischer Beziehung

7. Herr Oberstabsartzt Dr. A. Weisbach bespricht die der Anthropologischen Geselshaft zur Publikation überstandte Arbeit des Herrn Hauptmanns HEINRICH HIMMEL:

Herrn Hauptmann H. HIMMEL'S sehr Verdienstvolle Arbeit umfasst eine Tabelle justicieller Daten, weiters einen lateinischen Bericht über die Katholiken der Herzegowina aus dem Ordensschematismus der Franziskaner im Jahre 1882, ferner „Sanitäres aus der Herzegowina“ von Regimentsarzt Dr. HERRMANN des 99. Infant.-Reg., worin der Autor die schlechten hygienischen Verhältnisse schildert, unter welchen die Ein gebornen leben, welche, daher sehr häuftig an Malaria, acuten Exanthemen, Syphilis und Verletzungen erkranken; – weiter eine Schilderung der Jugend dieses Landes vom Lehrer EMIL WOSKA, der dieselbe als folgsam, ehrerbietig, gelehrig, strebsam und intelligent bezeichnet; – endlich eine Beschreibung des Soldatenmateriales und des Volkes überhaupt von HIMMEL selbst, mit photographischen Aufnahmen von Katolischen, orthodoxen und mohamedanischen Hezegowinern.

In seinem geschichtlichen Ueberblicke das graue Alterthum mit seinen fast ganz unzuverlässigen Angaben über dieses Land nur kurz erwähnend, gibt er an, dass zu Ende des 6. Jahrhunderts die wahrscheinlich mit vielen Slaven gemischten Avaren dahin gekommen sind, gegen welche Kaiser Heraklius im Jahre 619 die chrobathen aus dem Karpathenlande berief, welche sich im Küstengebiete zwischen der Arsa und Cettina niederliessen, die Avaren vertreibend und theilweise auch in sich aufnehmend; so sollen die heutigen Liccaner Ueberbleibsel der Avaren sein.

Im Jahre 626 hiess derselbe Kaiser die Serben südlich von den Kroaten sich niederlassen: An der Narenta und auf den vorliegenden Inseln, im einwärts gelegenen Berglande, im Gebiete von Ragusa und Cattaro bis Antivari hinab, sammt dem Hinterlande.

So hätten diese Länder seitdem ihre ständige slavische Bevölkerung erhalten, freilich an der Küste der Adria italienischen Einflüssen ausgesetzt, während im Innenlande zeitweise auch magyarische Einwirkungen und schliesslich der osmanische Einfluss sich geltend machten.

HIMMEL findet den Herzegovzen sehr religiös, treu und redlich, mit hohem Familiensinne und vorzüglichen geistigen Eigenschaften ausgestattet, jedoch dabei misstrauisch; als Soldat ist er sehr ausdauernd und genügsam, zurückhaltend und wehleidig.

Den für uns wichtigsten Theil dieser umfangreichen, interessanten Arbeit bilden die an 180 Soldaten im Alter von 20–35 Jahren vorgenommenen Messungen nach VIRCHOWS'S, dem BROCA'schen ganz ähnlichen Systeme, welches 40 Maasse an jedem Individuum nebst Angaben über die Farbe der Haare und Augen und das Körpergewicht verlangt.

Nur wer selbst Lebende gemessen, kennt die dazu nothwendige, ermüdende Mühe, wird aber deshalb umsomehr eine solche Arbeit zu schätzen wissen.

Nach diesen Messungen erfreut sich der Hezegowiner bedeutenden Körperlänge von 1752 mm, womit er selbst seine westlichen Brüder, die Festlands-Dalmatiner (1708 mm WEISBACH) übertrifft.
Demgemäss gehören die Herzegowiner zu den Völkern grössten Schlages in Europa; ja sie überragen an Wuchs sämmtliche bisher hierauf untersuchten europäischen Völker.
Ihr Haupthaar ist, ganz ähnlich wie bei den Südslaven an den Gestaden der Adria, weit vorherrschend dunkel (169 = 93,8 %), indem die blonden Haare (11 = 6,1 %) nur verschwindend spärlich vertreten sind; eigenthümlicher Weise kam unter allen 180 Männern kein einziger rothhaariger vor. Das Dunkel der Haare beschränkt sich vorzüglich auf die Farbentöne hellbraun (52 = 28,8 %) und dunkelbraun (87 = 48,3 %), ohne die dunkelste Nuance, schwarz (30 = 16,6 %), mit einer ansehnlichen Zahl zu erreichen.
In Übereinstimmung mit den gleichen anderweitigen Erfahrungen verhält sich die Farbe der Augen anders, bei welchen wohl immer noch die dunklen Schattirungen (104 = 57,7 %), aber in bedeutend minderem Grade als bei den Haare, die lichten (76 = 42,2 %) überwiegen, worin sie mit den Adrislaven genau übereinstimmen.
Die blauen Augen (36 = 20 %) und die grauen (40 = 22,2 %) treten viel häufiger auf als die lichten Haare; die meisten Männer haben hellbraune (51 = 27,7 %) und dunkelbraune (53 = 29,4 %), die allerwenigsten schwarze Augen (nur 1 = 0,5 %).
An Körpergewicht erreichen sie 70,7 Kilo, die Dalmatiner blos 69, gehören also mit diesen zu den gewichtigsten Männern Europas.
Ihr Kopf ist 180 mm lang und 157 mm breit, also nach seinem Index von 872 ausgesprochen brachycephal bei dem geringen Umfange von 548 mm.
Die Höhe des Gesichtes beträgt 183 mm, dessen Breite zwischen den Jochbogen 144 mm, was einen Gesichtsindex von 786 ergibt.
Ihre Schultern sind ansehnlich breit (416 mm), der Brustkasten sehr geräumig (Umfang 901 mm) und die Arme 773 mm lang; der Oberarm (313 mm) ist ansehnlich länger als der Vorderarm (267 mm), die Hand 193 mm lang und 88 mm breit (Index 455).
Die untern Gliedmassen sind viel länger (902 mm) als die oberen, relativ zur Körperlänge (1000) deren Hälfte übersteigend (514), wogegen die Arme (441) unter der selben bleiben, also kurz, di Beine aber lang genannt werden müssen.
Ihr Oberschenkel (393 mm) ist bedeutend kürzer als der Unterschenkel (444 mm) – ganz im Gegensatze zum Baue des Ober- und Vorderarmes – der Fuss zehr lang (273 mm) bei mässiger Breite (104 mm); sein Längenbreitenindex (380) ist wie immer kleiner als jener der Hand.
Die zahlreichen Messungen sollen etwas später von mir bearbeitet und mit jenen anderer Völker eingehend verglichen und in den Mitteilungen veröffentlich werden.

Instructions for Contributors

Last updated October, 2016

The *Genetics and Applications* (G&A) is the official journal of the Institute for genetic engineering and biotechnology, University of Sarajevo. It is envisaged as an international journal issued twice a year in print format, publishing peer-reviewed articles of novel and significant discoveries in the fields of basic and applied genetics. Special issues or supplements may also be produced from time to time upon agreement with the Editorial Board.

Topics covered within *Genetics and Applications* (G&A) include:

- molecular genetics,
- cytogenetics,
- plant genetics,
- animal genetics,
- human genetics,
- medical genetics,
- population and evolutionary genetics,
- conservation genetics,
- genomics and functional genomics,
- genetic engineering and biotechnology, and
- bioinformatics.

The main article types include original research, short communication, review and letter to the editor. Authors are encouraged to submit complete, unpublished, original works that are not under review in any other journals. Acceptable papers are those that gather and disseminate fundamental knowledge in all areas of genetics.

Manuscripts should be prepared using a standard word processing programme, and presented in a clear readable format with easily identified sections and headings.

Manuscript layout directions

- Typed with 1.5 line spacing (A4 format) with numbered pages;
- Font Times New Roman 12 should be used for the text, and Times New Roman 11 for tables and references;
- The sections should typically be assembled in the following order: Title, Authors, Authors' full affiliations including department and post/zip codes, Corresponding author, Abstract, Keywords, Introduction, Material and methods, Results and Discussion, Conclusions, Acknowledgements, Conflict Of Interest, References, Tables, List of figure captions;
- Footnotes in the main text are to be avoided

Title

The title needs to be concise and informative. It should:
(i) arrest the attention of a potential reader scanning a journal or a list of titles;
(ii) provide sufficient information to allow the reader to judge the relevance of a paper to his/her interests;
(iii) contain no more than 170 characters including spaces.

Authors and affiliations

The names and affiliations of the authors should be presented as follows:
Example

Mirjana Beribaka[1*], Selma Hafizović[2], Amela Pilav[3], Mirela Džehverović[3], Damir Marjanović[2,4,5], Jasmina Čakar[3]

[1]*University of East Sarajevo, Faculty of Technology, Zvornik, Bosnia and Herzegovina*
[2]*University of Sarajevo, Faculty of Science, Sarajevo, Bosnia and Herzegovina*
[3]*Institute for Genetic Engineering and Biotechnology, Sarajevo, Bosnia and Herzegovina*
[4]*International Burch University (IBU), Sarajevo, Bosnia and Herzegovina*
[5]*Institute for Anthropological Research, Zagreb, Croatia*

**Correspondence: E-mail: beribaka@gmail.com*

Abstract (max 250 words)

The abstract should be complete and understandable with no references. It is important to attract the attention of potential readers. The context and the rationale of the study are presented succinctly to support the objectives. The experimental methods and main results are summarised but should not be overburdened by numerical or probability values. The abstract ends with a short and clear conclusion.

Keywords (3 to 5 words)

Keywords should be short and specific.

Introduction

The introduction briefly outlines the context of the work, presents the current issues that the authors are addressing and the rationale to support the objectives, and clearly defines the objectives. For hypothesis driven research, the hypothesis under test should be clearly stated.

Material and methods

Material and methods should be described in sufficient detail so that it is possible for others to repeat the experiment.

Results and discussion

This should present only the author's own results and observations on the results, their significance for the subject-matter in question, and a comparison with the results of other authors in the same field.

Conclusions

Provide a brief overview of the subject of the research, the methodology applied, and the results achieved with a brief commentary.

Acknowledgment

In this section, the authors may acknowledge (max two sentences) their support staff, their funding sources (with research funder and/or grant number), their credits to companies or copyrighted material, etc.

Conflict Of Interest

In this section authors should declare any conflict of interest

References

References should be cited in the text as follows:

"The procedure used has been described elsewhere (Green, 1978), "or "Our observations are in agreement with those of Brown and Black (1979) and of White et al. (1980),"or with multiple references, in chronological order: "Earlier reports (Brown and Black, 1979, 1981; White et al., 1980; Smith, 1982, 1984).... ". In the list of references papers should be given in alphabetical order according to the surname of the first author.

Journal article

Hodgkin AL, Huxley AF (1952a) The components of membrane conductance in the giant axon of Loligo. J Physiol (Lond) 116:473-496.

Hodgkin AL, Huxley AF (1952b) The dual effect of membrane potential on sodium conductance in the giant axon of Loligo. J Physiol (Lond) 116:497-506.

Book

Shibamoto T, Bjeldanes LF (1993) Introduction to food toxicology. Academic Press Inc, San Diego.

Chapter in a book

Rodricks JV (2009) Food. In: Lippmann M. (ed) Environmental toxicants: Human exposures and their health effects. John Wiley & Sons Inc, Hoboken, pp. 197-239.

Tables

Tables with title should be submitted within manuscript file after the Reference section.

Figures

Figures (including graphs) should be submited in jpg. format named by "Figure 1". Figure title for each figure should be listed in main document after Tables or References section. A legend must be supplied for each illustration.

Editorial Board

Conference Invitation

1st SouthEast European Ichtyological Conference 2017

27-29 September
Sarajevo

SEEIC 2017 provides an ideal opportunity for researchers from the Southeast Europe or those who are interested in the region's ichthyofauna to meet and share their research findings, ideas and proposals.

Peer-reviewed full-text papers from the conference will be published in the journals Acta Adriatica (marine ecosystems) and the Croatian Journal of Fisheries (freshwater ecosystems).

We cordially invite you to save the date and join us in Sarajevo in September 2017

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