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EVALUATION OF GENETIC DIVERSITY OF EDIBLE HONEYSUCKLE MONITORED BY RAPD IN RELATION TO BIOACTIVE SUBSTANCES

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ABSTRACT

The aim of this study was clarifying the relation between genetic diversity of edible honeysuckle (*Lonicera kamtschatica*) and the major group of biologically active substances as total polyphenols content (TPC) including antioxidant activity (AO). Fruits of edible honeysuckle becomes more and more popular, especially in Europe. The current status of research on polyphenolic compounds in the berries of edible honeysuckle and their biological effects, including recommended utilization, are reviewed.The biological material including 14 cultivars of the edible honeysuckle (´Zoluška´, ´Amfora´, ´Pruhonický 44´, ´Vasilijevsky´, ´Moskovskaja´, ´Vojtek´, ´Sinoglaska´, ´Altaj´, ´Lipnická´, ´Kamčadalka´, ´Sinaja Ptica´, ´Fialka´, ´Modrý Triumf´, and ´Leningradský velikán´) originated from Czech republic (Žabcice near Brno). The content of TPC and AO were determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysuckle acessions, too. DPPH method was used to analyze AO and Folin-Ciocalteu method was used to determine TPC. The results of experiment showed that the highest value of AO was determined at the cultivars ´Zoluška´ (81.04 mg.L^{-1}) and the lowest was measured in ´Kamčadalka´ (54.122 mg.L^{-1}). On the contrary, the highest content of TPC was determined at the cultivar ´Kamčadalka´ (51.09 mg.L^{-1}) and the lowest value was measured at the cultivar ´Pruhonický 44´ (21.65 mg.L^{-1}). Phylogenetic trees were similar in genetic distance. The content of TPC and AO were not statistically significant in relation to cultivar. The analyzed cultivars of the edible honeysuckle were separated in 4 clusters according to used primers. In both gel images, the amplicon size ranged from 100 to 1,500 bp. We found that genetic diversity was partially related to content of total polyphenolic substances and antioxidant activity. Based on phylogenetic trees we have stated that variety ´Lipnická´, ´Sinoglaska´, ´Altaj´, ´Leningradský velikán´, ´Modrý Triumf´, ´Sinaja Ptica´ and ´Kamčadalka´ were grouped in the similar cluster. The highest genetic distance was determined at the variety ´Vasilijevskaja´ and ´Amfora´. In the same way, there were variety ´Vojtek´, ´Fialka´ and ´Zoluška´.

Keywords: honeysuckle; RAPD; DPPH; Folin-Ciocalteu; total polyphenols content

INTRODUCTION

Fruits of edible honeysuckle, despite their valuable qualities have been less well-known fruits species in the territory of Slovakia. Edible honeysuckle come from the territory of the Russian Federation. From the point of view of the soil-climatic conditions of the locality, the al plants are not demanding. Among their precious properties in terms of growing conditions are high freezing resistance as well as resistance to diseases and pests (the incidence of plant diseases affected by diseases and pests is very low). Furthermore, their growing importance based on the early flowering period, which also result in earlier planting of the plants, thus significantly reducing the length of the growing season **(Matuškovič et al., 2003)**. Moreover, the fruit of edible honeysuckle have been rich in phenolic acids, flavonoids (quercetin, rutin, anthocyanins) and ascorbic acid content too **(Juríková et al., 2012)**.

The genetic aspect and content of biologically active substances of *Lonicera kamtschatica* varieties (Sevast.) Pojark, have been still only a little explored **(Naugžemys et al., 2014)**.

The existence of multiple taxonomic classifications means that several names are used for the same taxa. According to **Handa et al. (2006)**, *L. kamtschatica* is considered a separate species. Phylogenetic analyzes of cultured plants are very important in terms of their taxonomy.

Plants produce bioactive substances as secondary metabolites in their defense, which have considerable fungicidal, bactericidal and biocidal activity, such as e.g. protect the embryo from harmful UV radiation. The action of antioxidants in the human body protects the body from the effects of exogenous and endogenous free radicals. In addition to endogenous low-molecular-weight antioxidants (glutathione, uric acid, coenzyme Q, etc.), substances of natural origin are also at the center of attention, ie those substances that are taken up by the body through food. Above all, they are vitamins like C, E and carotenoids. Other polyphenolic substances, along with these, occur in

vegetables, fruits, teas, wines, and, last but not least, in aromatic and medicinal plants **(Kaczmarska et al., 2015)**.

Nowadays, plant breeding has been focused on producing large-fruited varieties with regular fertility and high polyphenolic content in combination with vitamin C. In recent years, different breeding programs on *Lonicera kamtschatica* were conducted in Europe, US and Canada **(Becker et al., 2019)**. Over the course of several millennia, refinement of crops has only been done by selecting the most viable and fastest growing plants. The selection should then influence on morphological and quantitative properties of crops. After identifying DNA as a carrier of heredity and describing its chemical structure, studies have focused on more detailed DNA properties, its association with enzymes that are present in cells of living organisms. Studies have also led to revelations of mechanisms such as a gene that is stored in a DNA molecule can encode a visually detectable attribute. The development of this scientific field has also had a profound impact on modern breeding methods **(Holubec et al., 2019)**. Authentication of raw plant materials are required and necessary for the standardization of functional foods and medicaments **(Heinrich, Švarcová and Valentová, 2008; Jiang et al., 2013)**. Identification of honeysuckle with DNA-based molecular tools has been used to obtain promising genotypes in terms of flavonoids, phenolic acid content and high antioxidant activity (DNA barcoding - **Sun et al., 2011**; ITS sequencing - **Hu et al., 2012)**. For example, characterization of *Lonicera caerulea* by ISSR markers **(Kaczmarska et al., 2015)**, specific SCAR markers developed from the high GC-RAMP-PCR products of *Lonicera japonica* **(Cheng et al., 2016)** or the quality marker concept and a set of integrated strategies used to improve the two chemical markers of *Lonicera japonica flos* (LJF) and *Lonicera flos* (LF) often confused in the management of chemical marker quality **(Ding et al., 2017)**.

The aim of this study was clarifying the genetic diversity of selected cultivars of edible honeysuckle in relation to content of the predominant group of the biologically active ingredients sumed up as total polyphenols content (TPC) and antioxidant activity (AO) of fruits.

Scientific hypothesis

The content of bioactive substances are determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysukle acessions, too.

MATERIAL AND METHODOLOGY

In this study, we evaluated the genetic variability of edible honeysuckers, the content of bioactive substances, and the relation between these two attributes.

Biological material - characteristics of the studied plants

The assayed biological material included the cultivars of species *Lonicera kamtschatica* (Sevast.) Pojark and *Lonicera edulis* Turcz. Ex Freyn. Plant material was originated from the Czech Republic. The experimental area Lednice (Czech Republic) is located at an altitude of 177 m above sea level with a long-term average annual temperature of 9.7 °C and an annual average rainfall of 525 mm. The warm weather at the end of March accelerated the onset of the growing season.

In the research, the following cultivars of edible honeysuckle were selected:

´Zoluška´, ´Amfora´, ´Pruhonický 44´, ´Vasilijevsky´, ´Moskovskaja´, ´Vojtek´, ´Sinoglaska´, ´Altaj´, ´Lipnická´, ´Kamčadalka´, ´Sinaja Ptica´, ´Fialka´, ´Modrý Triumf´, and ´Leningradský velikán´.

The collection of biological material necessary for the individual analyses was carried out from Žabčice- Brno, Czech Republic at the end of June. To obtain representative samples for the determination of the content of bioactive substances (TPC and AO), it was necessary to collect berries from different parts of the plants (i.e., top, middle and down). ´Altaj´ is a foreign-born variety bred by the crossing of *Lonicera kamtschatica* x *Lonicera turczaninowii*. The fruits are elongated with a pointed tip and weigh about 0.7 to 1 g. The colour of the fruit is dark blue and has a sweet-sour taste. ´Amfora´ is a self-pollinating variety created by loose pollination of the ´Roksana´ variety. The fruits have a smooth surface and weigh from 0.9 to 1.2 g. The colour of the fruit is purple and the fruit has a sweet and aromatic taste. The variety ´Fialka´ was achieved by the same way as the ´Amfora´ variety, the fruit are cylindrical with average weight 0.7 – 0.9 g. Fruits weigh about 0.8 g and have a sweet-sour taste. The variety ´Leningradský velikán´ is a partially self-pollinating variety with ovate fruit. Fruits are cylindrical in shape and reach a weight of about 1 g. They are dark blue with uneven surfaces and have a distinctive scent. Together with cultivar Sinnaja Ptica were obtained from Research Institut in Sankt Peterburg in 1999. ´Sinnaja Ptica´ fruit are oval shaped and medium sized 0.7 – 0.9 g.´Kamčadalka´ belongs to the first generation of bred varieties in the Russian Bakcari breeding station. The fruit are elongated-oval 0.7 – 0.9 g. Moskovskaja was the next accesiom originated from Russian Federation and cultivated in Žabčice in 2011. ´Pruhonicky 44´ is represented genotype achieved from botanical expedition of researches from VŠÚO (Czech Republic) in Kamčatka, the fruit are dark blue with smooth surface, the average weight is 0.7 g. ´Sinaja Ptica´ and ´Zoluška´ were selected in NII of (Novosibirsk Institut of fruit production in Sibir). The fruit of ´Sinaja ptica´ are dark grey with smooth surface, elongated, medium sized 0.87 g. ´Zoluška´ fruit are medium sized the average weight of fruit is 0.71 g. ´Sinoglaska´ represented cultivar with sour taste and elongated oval fruit, on the other hand ´Vasiljevsky´ can be characterized by cylindrical sweet fruit 0.7 – 0.9 g. ´Vojtek´ represented Polish variety with tart sweet taste of fruit 1 – 1.5 g reminded blueberry. ´Lipnická´ is cultivar of *Lonicera kamtschatica* originated from Czech Republic with cylindrical fruit medium weighted 0.7 – 0.9 g.

DNA extraction

For the molecular laboratory for the RAPD method, the leaves were harvested without visible damage. DNA from fresh young plant leaves was isolated using the CTAB protocol by **Rogers and Bendich (1994)**.

RAPD amplification

RAPD-PCRs were carried out in volumes of 15 µL, containing 50 ng of DNA, 7.5 µL Combi mastermix, 1 µL primer and 5.5 µL water.

The thermal cycler (My Cycler BioRad) was programmed for one cycle of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, and finally by one cycle of 5 min at 72 °C.

Amplicon analysis

Amplification products were separated by electrophoresis (BioRad) in 6% PAGE (30% acrylamide, 5xTBE, 10% APS, TEMED). Gels were stained with GelRed, visualized by Transilluminator UVP with documentation system G-Box SynGene and analytic software GeneSnap, SynGene. Marker GeneRulerTM DNA Ladder Mix (MBI Fermentas) was used to determine the size of the DNA fragments. Fourteen plants including *Lonicera* were analysed using 2 RAPD primers (ACCGCGAAGG and GGACCCAACC). DNA fragments detected not in all accessions profiles were considered as polymorphic. Amplicon analysis were similar as in study **Vivodík et al. (2019)**.

Fihure 1 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Altaj´ **(Mlček, 2013)**.

Fihure 2 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Fialka´ **(Mlček, 2013)**.

Fihure 3 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Kamčadalka´ **(Mlček, 2013)**.

Fihure 4 Fruits of honeysuckle *Lonicera kamtschatica* cultivar ´Leningradský velikán´ **(Mlček, 2013)**.

Determination of content of total polyphenols content (TPC) and antioxidant activity (AO)

Determination of antioxidant activity by DPPH method, which consists in reaction of test substance with DPPH (stable free radical 1,1-diphenyl-2-picrylhydrazyl) by method **Quiros et al. (2010)**. The measurement took place at $\lambda = 515$ nm.

Determination of total polyphenolic content by Folin-Ciocalteu method was performed with Folin-Ciocalteu reagent, 1.5 mL 20% Na_2CO_3. Methodology for the determination of this method is implemented by **Paulová, Bochořáková and Táborská (2004)**.

Statistic analysis

Gel images were analyzed using Gel-Pro Analyzer 2010a (Media Cybernetics, L.P, USA). The values we acquired were recalculated using Neighbor-Joining using PHYLIP software (University of Washington, Seattle, version 3.696). Through the clustering method, we transformed data to create a phylogenetic tree. Distancematrix is used as input. In this method we also used the Q-matrix method. For this study there has been used UPGMA statistic method by **Nei and Li (1979)**. We used the Dendroscope V 3.5.9 software to construct the dendrogram of analysed accessions. The values of AO and TPC content were analyzed by statistical methods correlation analyse and ANOVA. The results have been shown in constructed trees.

RESULTS AND DISCUSSION

In the study **Kucharska et al. (2017)** were identified 50 compounds included 15 iridoids, 6 anthocyanins, 9 flavonols, 2 flavanonols (dihydroflavonols), 5 flavones, 6 flavan-3-ols, and 7 phenolic acids. 8-*epi*-Loganic acid, pentosyl-loganic acid, taxifolin 7-*O*-dihexoside, and taxifolin 7-*O*-hexoside were identified in honeysuckle berries for the first time.

Our results of determination of AO and TPC of the selected 14 cultivars of edible honeysuckle are given in Figure 5 and 6.

The TPC values for different *Lonicera kamtschatica* cultivars originated from territory of Czech Republic ranged from 57.50 to 90.30 mg/GAE/l FW **(Rop et al., 2011a)** that represented lower values with assayed cultivars in the same conditions of cultivation . The highest content of TPC was determined at the cultivar ´Kamčadalka´ (51.09 $mg.L^{-1}$) and the lowest value was measured at the cultivar ´Pruhonický 44´ (21.65 $mg.L^{-1}$).

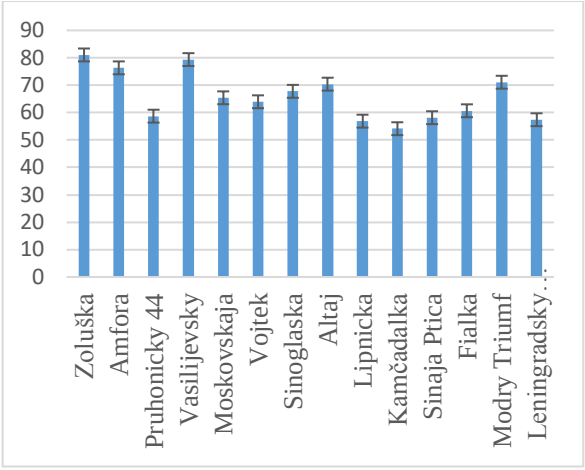

Figure 5 Average AO content (equivalent to TROLOX mg.l^{-1}).

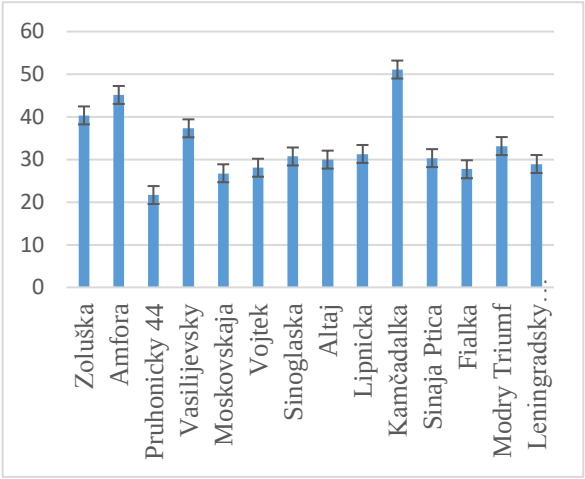

Figure 6 Average content of PP (polyphenols to gallic acid mg.l^{-1}).

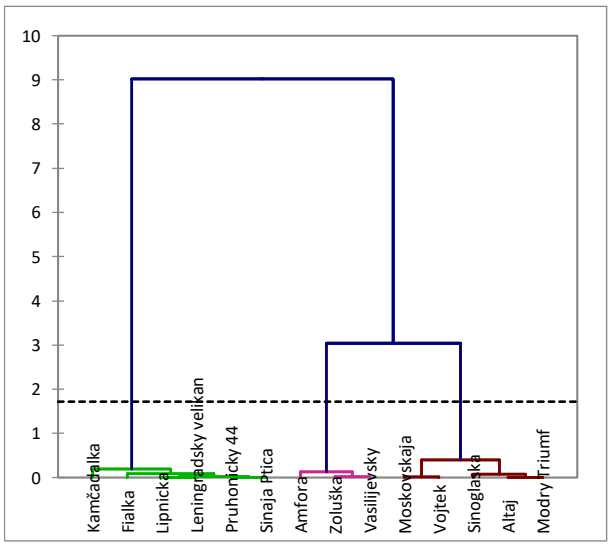

Figure 7 Average AO content (equivalent to TROLOX mg.l^{-1}). Note: According to AO cultivars were separated into 3 class:
Cluster 1: ´Zoluška´, ´Amfora´, ´Vasiljevský´;
Cluster 2: ´Pruhonický 44´, ´Lipnická´, ´Kamčadalka´, ´Sinaja Ptica´, ´Fialka´, ´Leningradský velikán´;
Cluster 3: ´Moskovskaja´, ´Vojtek´, ´Sinoglaska´, ´Altaj´, ´Modrý Triumf´ .

The results of determination of AO showed that the highest value was determined at the cultivar ´Zoluška´ (81.04 mg.L^{-1}) and the lowest values were measured at the cultivar ´Kamčadalka´ (54.122 mg.L^{-1}) and Lipnicka (56.88 mg.L^{-1}). Similarly, in the study research of **Juríková et al. (2014)** compared Russian cultivars of *Lonicera kamtschatica* ´Lipnická´achieved the lowest value of AO. In fresh honeyberry fruits, high values of analyzed bioactive compounds (vitamin C, TPC, TFC, TNFC and TAH) and antioxidant capacity were observed in the study of **Žlabur et al. (2019)**. The total phenol content (TPC) in FHs samples was 6.209 g GAE.100g^{-1} DM.

Average value of total content of AO was 65.8 mg.L^{-1}. Average value of total content TPC reached up 33.025 mg.L^{-1}. **Gazdík et al. (2008)**, who studied 21 clones of *Lonicera kamtschatica*, pointed to a statistically significant positive weak correlation between anthocyanin and ascorbic acid content in samples studied in 2008.

As Figure 3 and figure 4 showed antioxidant activity (AO) and total content of polyphenols (TPC) were similar except for ´Kamčadalka´, ´Lipnická´. They were extended separated as group 5. In the same way, ´Sinoglaska´, ´Altaj´, and ´Modrý Triumf´ were extracted as group 4. In the same way ´Vasiljevský´ and ´Leningradský velikán´ created the separated groups in TPC content in the study of **Sochor et al. (2014)**. On the other hand, ´Amfora´, ´Vasiljevský´ were group into one cluster together with ´Leningradský velikán´ and ´Altaj´.

Because of high degree of similarity in AO and TPC content, the correlation analysis was provided as well. By using statistical method correlation we have found that all represented pairs of values lay on a single line and the function has a rotating character. The coefficient was equal to +1, thus showing a greater degree of interdependence, and the observed values reflect a higher degree of interdependence. It means that there has been positive correlation between the content of TPC and AO (r = 1). In the same way **Matuškovič et al. (2009)** found a statistically significant positive strong correlation in the same samples of *Lonicera kamtschatica* cultivars in 2009. **Sochor et al. (2014)** found out the statistically significant correlation between TPC and AO assayed 20 cultivars of *Lonicera kamtschatica* originated from territory of Žabčice (r^2 = 0.998). Another study by **Rop et al. (2011b)** using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test in particular cultivars of *Lonicera kamtschatica* introduced into the conditions of the Czech Republic pointed to high antioxidant activity of fruit ranged from 6.59 – 10.17 g of ascorbic acid equivalent/kg of fresh mass significantly correlated to TPC content. The antioxidant activities were well correlated with the total phenolic and total anthocyanin contents in the study **Zhao et al. (2015)**.

The analyzed cultivars of the edible honeysuckle were separated in 4 clusters according to used primers. In both gel images, the amplicon size ranged from 100 to 1,500 bp. In the similar way, both phylogenetic trees were similar in genetic distance. Based on phylogenetic trees we have stated that variety ´Lipnická´, ´Sinoglaska´, ´Altaj´, ´Leningradský velikán´, ´Modrý Triumf´, ´Sinaja Ptica´ and ´Kamčadalka´ were grouped in the similar cluster. The highest genetic distance was determined at the variety ´Vasilijevskaja´ and ´Amfora´. In the same way, there were variety ´Vojtek´, ´Fialka´ and ´Zoluška´.

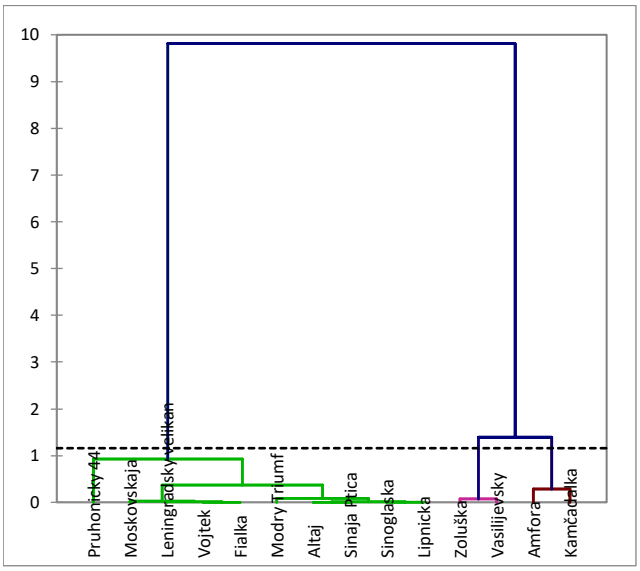

Figure 8 Average content of TPC (polyphenols to gallic acid mg.L^{-1}). Note: Based on TPC content the cultivars were distinguished into 5 clusters:
Cluster 1: ´Zoluška´, ´Amfora´, ´Vasiljevský´;
Cluster 2: ´Pruhonický 44´, ´Sinaja Ptica´, ´Fialka´, ´Leningradský velikán´;
Cluster 3: ´Moskovskaja´, ´Vojtek´;
Cluster 4: ´Sinoglaska´, ´Altaj´, ´Modrý Triumf´;
Cluster 5: ´Lipnická´, ´Kamčadalka´.

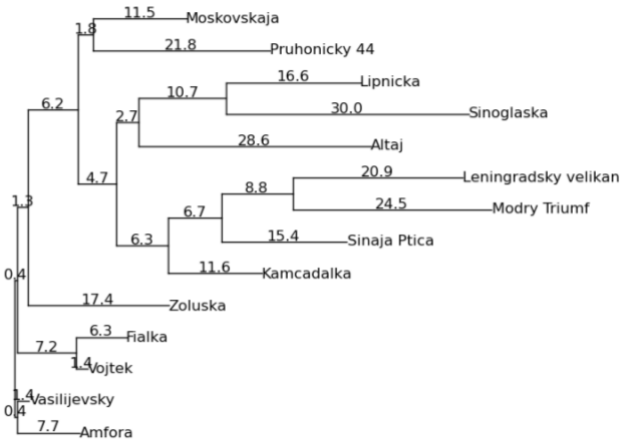

Figure 9 Phylogenetic tree of 14 varieties *Lonicera sp.* (used primer ACCGCGAAGG).

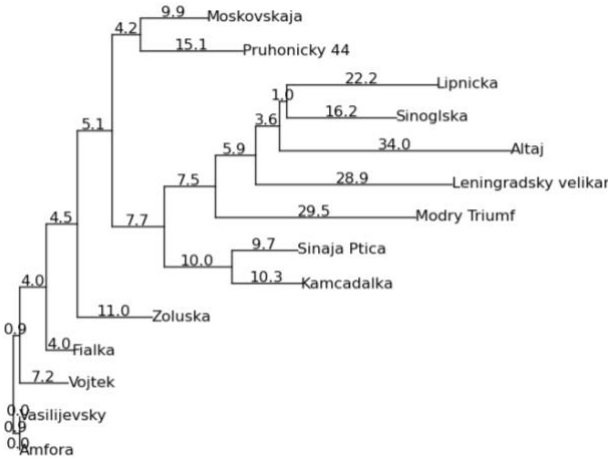

Figure 10 Phylogenetic tree of 14 varieties *Lonicera sp.* (used primer GGACCCAACC).

We found out there has proved only partial similarity in relation between dendrograms of total antioxidant activity and polyphenolic content compared to phylogenetic trees. The polyphenols content and antioxidant activity of *Lonicera* fruit has been partially influences by genetic background of plants and conditions of cultivation **(Juríková et al., 2012)** that has been proved in our study too. This examination was proved by ANOVA evaluation, in which the content of TPC and AO were not statistically significant in relation to cultivars ($p \geq 0.05$). A combination of enviromental conditions (temperature, precipation, light intensity led to different accumulation of secondary metabolites in honeyberry fruit **(Senica et al., 2018)**.

The markers in RAPD allow the identification of species or isolates, and the construction of dendrogram from the computed distances **(Williams et al., 1990)** although there are some problems with this technique. One of the main limitations of this technique is the low level of repeatability of band pattern if the amplification reactions are not optimized **(Fu et al., 2013)**. Usually the number of bands produced by RAPD primers is independent of the size of the genome, with an average number of five bands per reaction. Genetic diversity of different *Lonicera* species influenced by geographic distance, has been reported previously **(Lima et al., 2011)**. However, to characterize the genetic feature of *L. kamtschatica*, more samples from a wide range of geographical regions should be compared. Also other molecular markers may also be combined with RAPD analysis for more accurate authentications **(Fu et al., 2013)**.

CONCLUSION

The content of TPC and AO were determined by location and its soil-climatic conditions and these environmental circumstances determines the RAPD profiles of analysed honeysukle acessions, too. Based on constructed phylogenetic trees and dendrograms, there was high statistically approved results. The content of TPC and AO was not statistically significant in relation to cultivars ($p \geq 0.05$). We found that genetic diversity was partially related with content of total polyphenolic substances and antioxidant activity of fruit.

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