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Genetic structure of wild raspberry populations in the Central Balkans depends on their location and on their relationship to commercial cultivars

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ABSTRACT

The red raspberry (*Rubus idaeus* L.) is one of the most important fruit species in Serbia; there are currently approximately 11000 ha under cultivation. Wild red raspberry populations can be an important source of genes for breeding new raspberry varieties. This study was carried out to determine the genetic variability of wild populations, as well as their relationship with the most common cultivated cultivars using amplified fragment length polymorphism (AFLP) markers. In this study were included 128 individuals from seven wild populations and seven cultivar specimens from a nursery ('Mekeer', 'Willamette', 'Polka', 'Polana', 'Loganberry', 'Tayberry', 'Black Jewel'). Four AFLP combinations yielded a total of 247 polymorphic bands in 135 *R. idaeus* specimens. Analysis of AFLP markers showed that the cultivars are clearly distinct from wild populations. Population P4 (Mt. Ozren) had the highest gene diversity, the highest number of private markers and the highest rarity index. Analysis of molecular variance among and within wild populations showed that the most of the genetic diversity was attributable to differences between individuals within populations. A Mantel test showed that differences in altitude play the crucial role in structuring of the genetic diversity of red raspberry populations. Altitude contributed more to the population differentiation than spatial distance. Populations of wild raspberry from the territory of Serbia are characterized by high diversity, and should be protected and used as a resource in selection and breeding processes for the production of new varieties, with improved properties in terms of better yield and stronger resistance to external factors.

1. Introduction

The genus *Rubus* belongs to the family Rosaceae, and it is among the most diverse genera with over 700 species. There are 13 subgeneric categories described and the three largest are: *Idaeobatus* (raspberries), *Malachobatus* (primarily Asian species), and *Rubus* (= *Eubatus* Focke; blackberries) (Weber, 1995). *Rubus idaeus* belongs to subgenus *Ideobatus* which comprises approximately 200 species, and has a northerly distribution principally in Asia, eastern and southern Africa, Europe and North America. The wild raspberry is a perennial shrub with a height of 100–150 cm. The stem is erect, cylindrical and greyish, with a number of small thorns on the surface. The leaves are pinnate, of 5–7 leaflets, or sometimes of 3, glabrous on the surface and very hairy on the abaxial side. The terminal leaflet is oblong or ovate and shallowly lobed, whereas stipules are fibrous or hairy. The cyme inflorescences are made of flowers that are usually lying down, composed of narrow white, glabrous and whitish petals. The fruit is pale pink or light orange and seed is readily dispersed by birds and mammals, which can potentially generate high levels of gene flow and little population structuring (Graham et al., 2009). Under natural conditions, the red raspberry is almost exclusively self-incompatible (Keep, 1968). In the Balkans, the wild raspberry mostly grows at altitudes above 1000 m a.s.l., occurring on the edge of beech forests, in extensive orchards and vineyards, hedges, pastures and abandoned meadows and other undermanaged habitats. Examination of existing comprehensive Balkan vegetation databases (Aćić et al., 2012) shows that the distribution of the wild raspberry is in the form of fragmented populations, which occur in groups of approximately 10 m^2 areas, being distant from the next group in length from hundreds of metres up to 10 km (Marshall et al., 2001).

The basic chromosome number of *Rubus* is seven. Most raspberries are diploids (2n = 2x = 14) and have a very small genome (275 Mb), unlike blackberries where chromosome numbers are from

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2n = 2x = 14 to 2n = 12x = 84 (Daubeny, 1996).

Commercial *Rubus* fruit crops include the red (*Rubus idaeus* L.), black (*R. occidentalis* L.) and purple (red and black raspberry hybrid) raspberries, blackberries (*R. fruticosus* L.), Andean blackberries (*R. glaucus* Benth.) and cloudberries (*R. chamaemorus* L.) (Thompson, 1997). Most of the cultivars grow best and provide greater economic returns when cultivated in areas with mild winters and long dry summers.

Current raspberry cultivars are derived from the European red raspberry (*R. idaeus* L.), North American red raspberry (*R. strigosus* Michx.), the black raspberry (*R. occidentalis* L.) and the purple raspberries (*R. neglectus* Peck) which are hybrids between red and black raspberries. Most commercial varieties of raspberry are hybrids developed by breeding of the wild ecotypes (Çekiç and Özgen, 2010). Domestication of the red raspberry occurred in the past 400 to 500 years.

The most important cultivar in Serbia is 'Willamette', with about 90% of total production, followed by 'Meeker', with about 3–5% (Nikolić et al., 2008). Other cultivars, such as 'Polka' and 'Polana', are starting to gain in popularity among growers, due to increased consumer demand. According to data for the period 2010–2015, raspberry is the leading fruit species in Serbia according to export value of 241 756 USD (http://www.trademap.org/Index.aspx). There are about 11041 ha under cultivation, and this area is increasing. According to the FAO data from 2016 (http://www.fao.org/faostat/en/#data), Serbia is among the five biggest world raspberry producers with annual production of more than 62,000 tonnes.

Wild populations of *R. ideaus* are an important source of genes for breeding new raspberry varieties (Marshall et al., 2001). Nearly 60 major gene traits, important for the selection process, have been reported in this species (Jennings, 1988). The most significant include resistance to raspberry root rot (*Phytophthora rubi*), raspberry spur blight (*Didymella applanata*) and large raspberry aphid (*Amphorophora ideai*). Some of the most important characteristics of the fruit are: size and shape, firmness, colour, taste, aroma, suitability to different processing methods, postharvest response and ease of harvest (http://institut-cacak.org/eng/malina.html). In the development of improved varieties, the main traits in breeding are: hardiness, productivity, disease resistance, fruit size and firmness. There are reports on using *R. idaeus* as a model plant to develop and apply genetic techniques in perennials, particularly members of the family Rosaceae (Graham et al., 2003).

The aim of this study was to assess the genetic variability of natural populations of wild raspberry as an important and under-researched natural resource of high economic interest, and as highly valued material for selection and breeding programs. In addition, the most used commercial varieties in Serbian raspberry production were included in the AFLP analysis to estimate the possible relations and position regarding wild populations from the studied region.

2. Materials and methods

2.1. Plant material

In this study we included 135 individuals (fresh leaves) of *R. idaeus*, out of 128 were from 7 wild populations (Table 1) and 7 specimens were cultivars from the nursery “Superior D.O.O. “Velika Plana (C1 'Mekeer'; C2 'Willamette'; C3 'Polka'; C4 'Polana'; C5 'Loganberry'; C6 'Tayberry'; C7 'Black Jewel').

The collecting sites were selected using the criterion of richness of the natural populations of the wild raspberry, being determined by the analyses of existing floristic and phytogeographical data and insight in comprehensive vegetation data bases for the central Balkan (Aćić et al., 2012). The selected sites belong to three spatial groups: the Western group (Mt. Željin, Mt. Goč and Mt. Studena planina), the Eastern group (Mt. Ozren and Mt. Stara planina) and the Central group (Mt. Kopaonik and Mt. Golija), and to two altitude groups – the Mountainous region (Mt. Goč, Mt. Studena planina and Mt. Ozren) and the Alpine region (Mt. Željin, Mt. Kopaonik, Mt. Golija and Mt. Stara planina). The sampling sites were selected so as to represent the populations that were uniformly distributed across the species range, and at two different altitudes. Genetic differentiation among natural populations of wild raspberry was studied to determine the possible existence of genetic isolation either by geographic (spatial) position, or altitude.

Specimens from wild populations were collected and silica dried during field study in the period June - August 2016, while cultivars were collected in June 2017. Wild specimens were collected in way that ensured individual plants were at least 30 m apart from each other in order to avoid sampling of different ramets of the same genet. Collected samples were determined according to Bulatović (1972) and Flora Europea. Voucher specimens are deposited at the Herbarium of Department of Botany, Faculty of Agriculture, University of Belgrade.

2.2. DNA isolation and AFLP analysis

Total genomic DNA was extracted from 20 mg of silica gel dried leaves using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). DNA concentrations were measured using a Qubit Fluorometre (Invitrogen®). The AFLP analysis was performed according to the methods described by Vos et al. (1995), but with several modifications (Carović-Stanko et al., 2011). The restriction digestion and adapter ligation, pre-amplification and selective amplifications were performed in a GeneAmp® PCR System 9700 (Applied Biosystems®). Four primer combinations were selected for amplification (VIC-*Eco*RI-ACG + *Tru*1I-CGA; NED-*Eco*RI-AGA + *Tru*1I-CAC; FAM-*Eco*RI-ACA + *Tru*1I-CAC; PET-*Eco*RI-ACC + *Tru*1I-CGA). The prepared samples were detected using an ABI3130xl Genetic Analyzer (Applied Biosystems®). The presence or absence of fragments was scored on the chromatograms with GeneMapper 4.0 software (Applied Biosystems®). All fragments between 50 and 500 bp were scored. Peaks were automatically transposed into a binary matrix. When a peak height exceeded the absolute value of 50, as adjusted in the peak amplitude threshold settings of GeneMapper 4.0, the peak was scored as present (1); otherwise, it was scored as absent (0).

2.3. Data analysis

Molecular diversity of the wild red raspberry populations was assessed by calculating the proportion of polymorphic markers (%P), the Shannon’s information index (*I*), the number of private markers (N_{pr}) and the rarity index (RI). Shannon's information index was calculated as $I = -\Sigma\ (p_i\ log_2\ p_i)$, where p_i is the phenotypic frequency (Shannon and Weaver, 1949). The frequency down-weighted marker values (*DW*; Schönswetter and Tribsch, 2005) were calculated using AFLPdat (Ehrich, 2006). The values were range standardised to obtain the rarity index (RI; Winkler et al., 2010). The proportion of polymorphic markers (%P), the Shannon’s information index (*I*) and the number of private alleles (N_{pr}) were calculated also for a group of red raspberry cultivars.

Allelic frequencies at AFLP marker loci were calculated from the observed fragment frequencies, using the Bayesian approach proposed by Zhivotovsky (1999) as implemented in AFLP-Surv v. 1.0 (Vekemans et al., 2002) assuming Hardy-Weinberg equilibrium justified by the outcrossing nature of *R. idaeus*. The allelic frequencies were used in the analysis of genetic diversity within (H_E) and between wild populations following the treatment of Lynch and Milligan (1994). The population genetic structure was described in terms of the total gene diversity (H_T), the average gene diversity within populations (H_W), the average gene diversity among populations in excess of that observed within populations (H_B), and Wright's F_{ST} statistics.

Pairwise genetic distances between individual plants were calculated using Dice’s coefficient (Dice, 1945). Principal co-ordinate analysis (PCoA), based on Dice's distance matrix, were performed using PAST version 2.01 (Hammer et al., 2001) to visualize the relationships

Table 1
Sampling sits of seven wild red raspberry populations from Serbia.

ID	Population	Latitude (N)	Longitude (E)	Spatial group	Altitude (m a.s.l.)	Altitude group
P1	Mt. Goč	43.57	20.73	S1	675	A1
P2	Mt. Studena planina	43.52	20.64	S1	983	A1
P3	Mt. Željin	43.47	20.83	S1	1357	A2
P4	Mt. Ozren	43.37	21.53	S2	931	A1
P5	Mt. Stara planina	43.36	22.58	S2	1710	A2
P6	Mt. Golija	43.19	20.25	S3	1432	A2
P7	Mt. Kopaonik	43.18	20.50	S3	1985	A2

Spatial group: S1 Western, S2 Eastern and S3 Central region.
Altitude group: A1 Montainous and A2 Alpine region.

between individuals.

Standard pairwise genetic distances between wild populations were calculated according to Nei (1972) and a Neighbor-net diagram was constructed from genetic distance matrix using SplitsTree 4 (Huson and Bryant, 2006). Bootstrap values (Felsenstein, 1985) were based on 1000 replicates generated using AFLP-Surv and subsequently used in NEIGHBOR and CONSENSE programs of the PHYLIP ver. 3.6b software package (Felenstein, 2004).

To assess further the relationships among cultivars a neighbour joining tree based of Dice's distance matrix was constructed. The tree was rooted using wild individuals. Statistical support of the branches was tested with bootstrap analysis using 1000 replicates (Felsenstein, 1985). The calculations were made using PAST version 2.01 (Hammer et al., 2001).

The presence of distinct evolutionary clusters among individuals has been evaluated using a Bayesian approach as implemented in BAPS 6.0 (Corander et al., 2003). The first analysis was carried out by inclusion of cultivars, whereas the second and third were performed using only wild populations. The second analysis was conducted without the geographic coordinates of populations used as an informative prior ('*Clustering of individuals*'), while for the third we used this prior ('*Spatial clustering of individuals*'). BAPS was run with the maximal number of clusters (K) set to 20 and each run was replicated 10 times. The best value of K was estimated using the log marginal likelihood values of the best partitions, and the distribution of posterior probabilities for different K values. Results of the mixture analysis were used as input for population admixture analysis, with the default settings, in order to detect admixture between clusters.

The analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to partition the total AFLP diversity (A) Among groups (wild vs. cultivated), among populations within groups and within populations, (B) among and within wild populations, (C) among three spatial groups (wild populations only), among populations within groups and within populations, and (D) among two altitude groups (wild populations only), among populations within groups and within populations. The information on assignment of populations into spatial and altitude groups is given in Table 1. The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations using Arlequin ver. 3.5.2.2 (Excoffier and Lischer, 2010). Pairwise comparisons between populations that were examined with AMOVA resulted in φ_{ST} values that were equivalent to the proportion of the total variance that was partitioned between the two populations.

We computed and tested the correlations between (A) the matrix of the natural logarithm of geographical distances (in km) between pairs of wild populations and the matrix of pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios and (B) the matrix of altitude differences (in m) and the matrix of pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios. In addition, a three-way Mantel test (C) was applied between the matrix of altitude differences and the matrix of pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios while accounting for geographical distances among populations. The significance level was assessed after 10,000 permutations as implemented in NTSYS-pc Ver. 2.02 (Rohlf, 1997).

3. Results

According to the geographical location of populations, all collected wild populations can be grouped in three spatial and two altitude groups. Spatial group 1 (Western) consist of populations P1, P2 and P3, spatial group 2 (Eastern) of P4 and P5 and spatial group 3 (Central) consist of P6 and P7. Depending of altitude, P1, P2 and P4 are in altitude group 1 (below 1000 m a.s.l. – Mountainous region) and P3, P5, P6 and P7 in group 2 (above 1000 m a. s. l. – Alpine region) (Table 1).

Four AFLP combinations yield a total of 247 polymorphic bands in 135 *R. idaeus* specimens. Proportion of polymorphic markers (%P) was similar across wild populations with the highest in P2 (68.02%) and the lowest in P3 (58.30%). Gene diversity (H_E) ranged from 0.152 (P6 and P7) to 0.179 (P4). The total diversity (H_T) was 0.167, the average gene diversity within populations (H_W) was 0.161 and the average gene diversity among populations (H_B) was 0.006. Differentiation among populations was low (F_{ST} = 0.038) but highly significant ($P < 0.0001$). Population P4 had the highest gene diversity (H_E = 0.179), the highest number of private markers (N_{pr} = 7) and the highest rarity index (RI = 0.374). RI values were positive in all mountainous populations (P1, P2, P4) and negative in all alpine populations with the exception of the population P5 (Table 2).

Average Dice's distance among wild specimens was 0.422, ranging from 0.062 to 0.720 while the average Dice's distance among cultivars was 0.463, ranging from 0.246 (between C1 'Mekeer' and C3 'Polka') to 0.682 (between C2 'Wilamette' and C7 'Black Jewel'). Average Dice's distance between wild and cultivated specimens was 0.544, ranging from 0.283 (between C1 'Mekeer' and an individual from population P5) to 0.822 (between C7 'Black Jewel' and an individual from population P5).

In the principal co-ordinate analysis (PCoA) based on Dice's distance matrix, the first three co-ordinates had eigenvalues higher than 1 and jointly explained 19.17% of the total variation. The first principal co-ordinate separated the individuals from mountainous populations from those belonging to alpine populations, while along both second and

Table 2
Molecular diversity of seven wild red raspberry populations from Serbia and the group of cultivars based on AFLP markers.

ID	Population/Group	*n*	%P	N_{pr}	N_{pr}'	*I*	H_E	RI
P1	Mt. Goč	22	66.40	0	0	0.375	0.159	0.085
P2	Mt. Studena planina	19	68.02	0	3	0.390	0.162	0.111
P3	Mt. Željin	15	56.28	0	5	0.365	0.161	−0.330
P4	Mt. Ozren	14	63.16	7	9	0.412	0.179	0.374
P5	Mt. Stara planina	19	67.21	1	1	0.394	0.161	0.080
P6	Mt. Golija	19	58.30	0	1	0.356	0.152	−0.150
P7	Mt. Kopaonik	20	61.54	0	0	0.360	0.152	−0.170
C	Cultivars	7	44.94	2	–	0.360	–	–

n - sample size; %P - proportion of polymorphic markers; N_{pr} - number of private markers; $N_{pr'}$ - number of private markers excluding the group of cultivars; *I* - Shannon's information index; H_E - gene diversity assuming Hardy-Weinberg equilibrium; RI - rarity index.

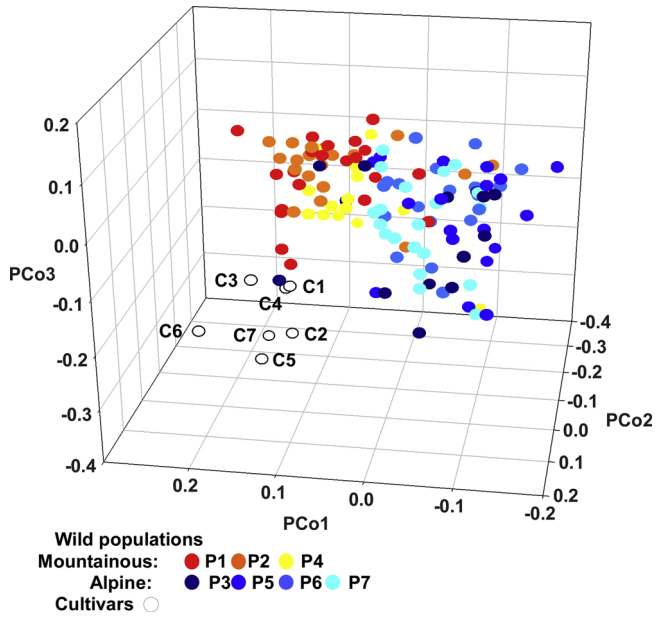

Fig. 1. Principal co-ordinate analysis (PCoA) based on Dice's distance matrix among individuals belonging to seven wild red raspberry populations and seven cultivars (C1 – 'Mekeer', C2 - 'Willamette', C3 – 'Polka', C4 – 'Polana', C5 – 'Loganberry', C6 – 'Tayberry', C7 – 'Black Jewel').

third principal coordinates the cultivars were clearly separated from wild specimens (Fig. 1).

Average Nei's genetic distance was 0.008, ranging from 0.001 (between populations P3 and P7) to 0.015 (between P4 and P7). Neighbour-net diagram based on Nei's genetic distance clearly separated mountainous populations from alpine populations with bootstrap support of 97% (Fig. 2). However, it indicated that the gene flow between mountainous and alpine populations takes place between mountainous populations P1 and P2 and alpine populations P3 and P7, as well as between mountainous population P4 and alpine population P6.

To assess the relationships among cultivars, a neighbour-joining tree based on Dice's distance was constructed (Fig. 3) revealing two clades, one consisting of cultivars C1 'Meeker', C2 'Willamette', C3 'Polka' and C4 'Polana' and the other of C5 'Loganberry', C6 'Tayberry' and C7 'Black Jewel'. However, clades were not supported (bootstrap values lower than 50%). Clustering of individuals including cultivars in BAPS

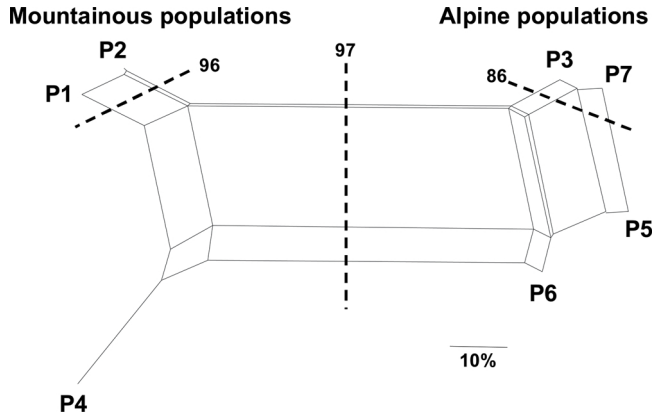

Fig. 2. Neighbor-net diagram based on Nei's genetic distances between seven red raspberry populations from Serbia. Bootstrap support values > 50% of 1000 replicates are given near the branches.

showed that the highest log marginal likelihood (-11,054.43) was obtained at K = 3 (Fig. 4A). The membership probabilities of all cultivars in cluster C were equal to 1 suggesting that the cultivars were clearly distinct from wild specimens. Additional analyses were performed excluding cultivars, without geographic coordinates used as informative prior (Fig. 4B) and with the geographic coordinates used as informative prior (spatial clustering; Fig. 4C). Both analyses yielded almost identical results. The best partitions (at K = 2) received log marginal likelihoods of -10,256.32 and -10,364.28, respectively. Cluster A included the great majority of individuals belonging to mountainous populations (P1, P2, P4) while cluster B included the great majority of individuals belonging to alpine populations (P3, P5, P6, P7). The average membership probabilities of populations to the best-scoring cluster ranged from 0.79 (P2 in cluster A in both analyses) to 1.00 (P6 in cluster B in both analyses).

Two-way AMOVA that included the group of cultivars revealed that 16.37% of genetic variance was explained by differences between wild and cultivated individuals [φ_{CT} = 0.164; $P(\varphi_{CT}) < 0.0001$] (Table 3). One-way AMOVA among and within wild populations showed that most of the genetic diversity was attributable to differences between individuals within populations (91.86%). However, the significant φ_{ST} value of the among-populations variance component suggested the existence of population differentiation.

Average pairwise φ_{ST} value between populations was 0.081, ranging from 0.007 (between populations P3 and P7) to 0.141 (between P4 and P7). All φ_{ST} values were significant ($P < 0.05$) except between populations P3 and P7. By grouping the populations into three spatial groups, the component of genetic variance attributable to differences among spatial groups was not significant [0.98%; φ_{CT} = 0.010; $P(\varphi_{CT})$ = 0.321]. On the other hand, the two-way AMOVA that included the analysis between two altitude groups revealed that the between-groups component of variance was significant [6.33%; φ_{CT} = 0.063; $P(\varphi_{CT}) < 0.0001$]. Moreover, the higher percentage of variation was attributable to between-groups differences, rather than to differences among populations within altitude groups.

The correlation between the geographical distances [*ln*(km)] and pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios was low (r = 0.136), and not significant according to a Mantel test (P_{Mantel} = 0.239) indicating the absence of the typical pattern of isolation-by-distance (IBD) among populations (Fig. 5A). On the other hand, a significant correlation was found between the differences in altitudes of the sampling sites (in m) and pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios (r = 0.589; P_{Mantel} = 0.015), suggesting that 34.7% of the genetic differentiation between populations could be explained by altitude differences (Fig. 5B). Similar results were obtained by testing the correlation between altitude differences and pairwise $F_{ST}/(1\text{-}F_{ST})$ ratios while accounting for geographical distances (r = 592; P_{Mantel} = 0.014; R^2 = 0.351) in a three-way Mantel test, confirming that the differences in altitudes played the crucial role in structuring of the genetic diversity of red raspberry populations (Fig. 5C).

4. Discussion

Rubus idaeus has been used for food and as a medicinal plant since ancient times, and knowledge of genetic diversity patterns can improve management and species conservation. AFLP based analyses proved to be useful in population structure studies and in inferring phylogenies at lower taxonomic level (Šoštarić et al., 2012; Peng et al., 2018). In this study we included seven wild populations, which can be separated into three spatial groups and two altitude groups, and seven commercial cultivars.

The lowest proportion of polymorphic bands was found in cultivars. Cultivation, through "domestication syndrome" (Harlan, 1992), and human selection and genetic drift, through bottleneck effects (Tanksley and McCouch, 1997), can lead to a loss of private alleles and a decrease

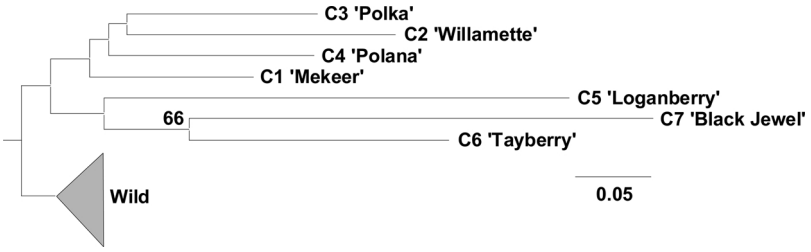

Fig. 3. Neighbor joining tree of red raspberry cultivars rooted by wild red raspberry individuals. Bootstrap support values > 50% of 1000 replicates are given above the branches.

in genetic diversity. Wright et al. (2005) reported single-nucleotide polymorphism (SNP) diversity of 774 gene fragments in 14 maize (*Zea mays* ssp. *mays*) inbred lines (representing modern maize) and 16 inbred teosintes (*Zea mays* ssp. *parviglumis*) and found 3463 SNPs in maize and 6136 SNPs in teosinte, that was generally consistent with a population bottleneck during the domestication of maize. In apricot (*Prunus armeniaca*), microsatellite markers showed a significant reduction in allelic richness and private allelic richness when North Mediterranean and Southwest Mediterranean regions were compared to Irano-Caucasian region (Bourguiba et al., 2012).

Principal co-ordinate analysis showed that cultivars are clearly distinct from the wild individuals, as PCo2 and PCo3 separate cultivars from wild populations. In Neighbour joining tree of red raspberry cultivars rooted by wild red raspberry individuals, two clades can be noted (Fig. 3), one comprising cultivars 'Mekeer', 'Willamette', 'Polka' and 'Polana', and the other comprising 'Loganberry', 'Tayberry' and 'Black Jewel'. The absence of high bootstrap support values can be explained by complex pedigrees of the cultivars (Dale et al., 1993). When cultivars are included in BAPS analysis, they all group together in cluster D (Fig. 4A). This clustering can be expected since ancestry of most cultivars is dominated by five parent cultivars, 'Lloyd George' and 'Pyne's Royal' derived from *R. idaeus*, 'Newburgh' from *R. strigosus* and 'Preussen' and 'Cuthbert' from crosses between *R. idaeus* and *R. strigosus* (Dale et al., 1993) and similar clustering of cultivars and wild populations can be observed in other species (Shim and Jørgensen, 2000).

The wild populations in this study showed similar levels of diversity across populations; in some cases, even if populations were strongly isolated, they still preserved similar levels of genetic diversity across the geographical range (Wróblewska, 2013). The highest diversity, most of the private markers and the highest rarity index was found in P4 from Mt. Ozren. These values indicate that P4 has been longer isolated, possibly with stable population dynamics over time, while other populations might be later formed (Yu et al., 2014). Shannon's information index (I) and gene diversity (H_E) were lower than average in populations belonging to altitude group above 1000 m a. s. l., except P5 from Mt. Stara planina, where I and H_E are higher than average (Table 2). Jump et al. (2006) analysed genetic diversity in three European beech (*Fagus sylvatica* L) populations from the sampling sites that included the upper treeline (1640 m a.s.l.; 70 samples), central forest area (1127 m a.s.l.; 69 samples), and the lower limit of *F. sylvatica* forest (992 m a.s.l.; 70 samples) and showed that the intrapopulation diversity decreased with altitude. By analysing 19 Japanese oak (*Quercus crispula* Blume) populations grouped into three altitude groups[A (< 1000 m a.s.l.; 4 populations; 78 samples), B (1000–1500 m a.s.l.; 8 populations; 157 samples), C (> 1500 m a.s.l.; 7 populations; 137 samples)], Ohsawa et al. (2007) reported that populations at intermediate altitudes had the highest expected heterozygosity and allelic richness, suggesting that the lower and higher populations had relatively little diversity because they were peripheral populations, growing in environments that were close to the environmental limits of the species.

In Principal co-ordinate analysis, PCo1 separates mountainous populations from alpine populations. Differentiation between

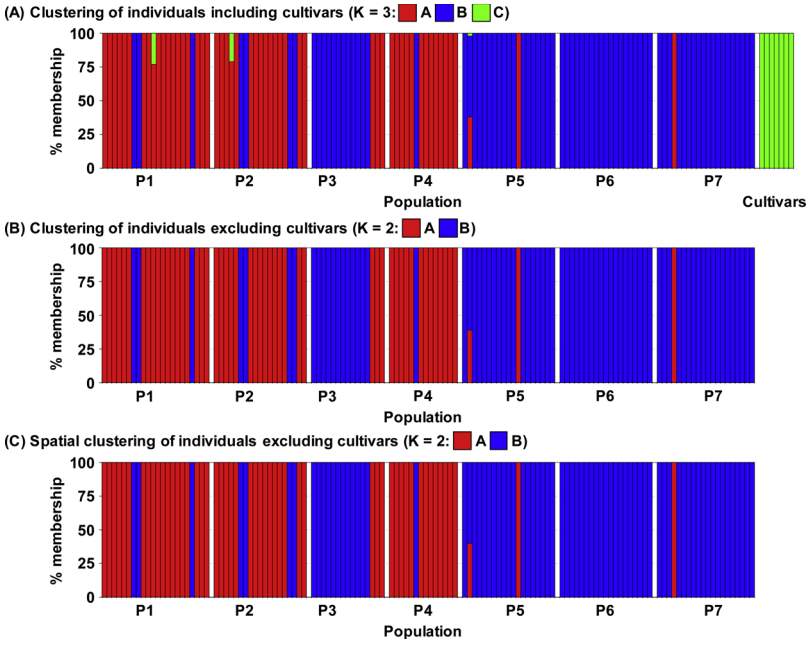

Fig. 4. Genetic structure of wild red raspberry populations and cultivars derived from Bayesian analysis using BAPS: (A) Clustering of individuals including cultivars, (B) Clustering of individuals excluding cultivars (without the geographic coordinates used as informative prior), and (C) Spatial clustering of individuals excluding cultivars (with the geographic coordinates used as informative prior).

Table 3
AMOVA analysis for the partitioning of AFLP diversity of red raspberry: (A) Among groups (wild vs. cultivated), among populations within groups and within populations, (B) among and within wild populations, (C) among three spatial groups (wild populations only), among populations within groups and within populations, and (D) among two altitude groups (wild populations only), among populations within groups and within populations.

Analysis	Source of variation	df	Variance components	Percentage of variation	φ-statistics	$P(\varphi)$
(A)	Wild vs. Cultivated	1	4.60	16.37	0.164	< 0.0001
	Among populations	6	1.90	6.75	0.081	< 0.0001
	Within populations	127	21.61	76.88	0.231	< 0.0001
(B)	Among wild populations	6	1.90	8.14	0.081	< 0.0001
	Within populations	121	21.48	91.86		
(C)	Among spatial groups	2	0.23	0.98	0.010	0.321
	Among populations	4	1.73	7.38	0.074	< 0.0001
	Within populations	121	21.48	91.65	0.084	< 0.0001
(D)	Between altitude groups	1	1.52	6.33	0.063	< 0.0001
	Among populations	5	1.03	4.28	0.046	< 0.0001
	Within populations	121	21.48	89.38	0.106	< 0.0001

The information on assignment of populations into spatial and altitude groups is given in Table 1.

mountainous and alpine populations is confirmed in Neighbour net diagram with high bootstrap support, as well as showing gene flow between neighbouring mountainous populations P1 from Mt. Goč and P2 from Mt. Studena planina and alpine populations P3 from Mt. Željin and P7 from Mt. Kopaonik. Gene flow between alpine population P6 from Mt. Golija and mountainous population P4 from Mt. Ozren can also be observed. Genetic differentiation along the altitudinal gradient can be observed in some other species, such as *Sophora davidii* (Li-Li et al., 2016) in which high genetic diversity at different altitudinal range could be attributed to various ecological factors. In numerous species it is thought that various factors, such as altitude, habitat fragmentation, location and population size, can have an impact on genetic diversity within populations. In *Salvia officinalis* (Rešetnik et al., 2016) analysis of SSR markers suggested that one of the possible evolutionary trajectories, after the last glacial maximum, is that species survived in several refugia exhibiting concurrent divergence into three genetic groups. SSR analysis of the 65 accessions of *Hordeum vulgare* L. ssp. *hexastichon* var. *nudum* showed geographical differentiation among the three subgroups (Feng et al., 2006).

BAPS analysis without cultivars grouped wild populations into two clusters. BAPS analysis of wild populations with or without spatially informative priors resulted in the congruent assignment of individuals to two clusters (Fig. 4B and 4C). The genetic structure of wild populations revealed by BAPS proved to be related, not to spatial groups, but to altitude groups. In *R. idaeus* this can be explained by difference in phenology between different altitudes and hence little opportunity of gene flow by pollen (Graham et al., 2003). Altitude population differentiation is also recorded in *Quercus petrea* in the French Pyrenees, where altitude contributed more to the population differentiation than spatial distance (Alberto et al., 2010).

AMOVA analysis indicates that most of the genetic diversity is found within populations, which is typical for allogamous species (de Souza et al., 2013). AMOVA analysis of wild populations indicated low population differentiation and that the partition among spatial groups was not significant, while partition among altitude groups was significant. Investigation of bud-burst and plant height in *Rubus idaeus* specimens from different altitudes in Scotland, suggested that those from higher altitudes tend to be differentiated from less exposed populations in respect of their late bud-burst and short growth (Jennings, 1964). Graham et al. (2003) used RAPD markers to test genetic differentiation in *R. idaeus* populations and obtained results showed that in *R. idaeus*, sites of genetically distinct populations can be grouped in mountainous, valley and alpine clusters. This also suggests higher gene flow between populations at the same altitude. A similar type of pattern was described in other plant species. In *Poa hiemata* genetic variation across six microsatellite markers revealed genetic structuring along altitudinal transects (Byars et al., 2009), while in *Oryza sativa* landraces AMOVA analysis based 48 SSRs and ten unlinked nuclear gene loci showed significant genetic differentiation among altitude zones (Cui et al., 2017). In *Fraxinus angustifolia* (Temunović et al., 2012) and *Tanacetum cinerariifolium* (Grdiša et al., 2014) results from populations in Croatia provided evidence that isolation by distance (IBD) as well as isolation by environmental distance (IBED) contributed to the genetic differentiation among populations. In this study a Mantel test showed absence of IBD while showed significant differentiation among *R. idaeus* populations along altitude.

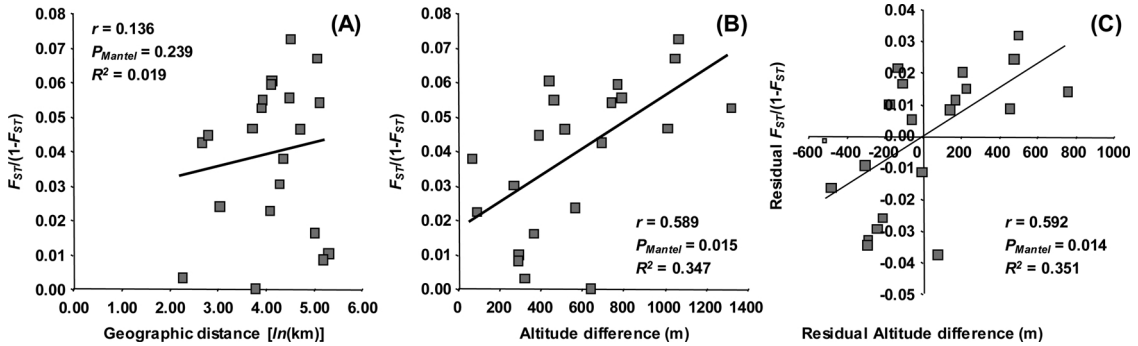

Fig. 5. Isolation-by-distance (IBD) and isolation by altitude in red raspberry populations from Serbia. Plots of simple and partial Mantel's tests showing the relationships between (A) geographic and genetic distances, (B) altitude and genetic distances, and (C) residual altitude and genetic distances by taking into account the geographic distances among seven red raspberry populations.

5. Conclusions

Genetic variability in wild populations is of significant importance for conservation of wild relatives, since they can be a valuable source of genes for future breeding programs, e.g. to improve tolerance to abiotic and biotic stress. Results show higher diversity in wild populations than in seven cultivars, as expected. It can also be concluded that altitude plays a more significant role in genetic separation of the populations than spatial distribution. The highest number of private markers, gene diversity and rarity index was found in P4 from Mt. Ozren, which is situated in the centre of the studied area, and that population can be recognized as the most distinctive from other populations. In the present study, AFLP markers proved to be useful in analysing the genetic diversity and population structure of wild and cultivated *Rubus idaeus* from the Central Balkans.

Declaration of Competing Interest

None.

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