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Viability and genetic diversity of *Populus nigra* population from riparian forest in SNR Gornje Podunavlje

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Abstract: *Populus nigra* L. is one of the rarest and most endangered tree species in Western and Central Europe. Its genetic diversity is of great importance in enabling a native riparian population to survive and reproduce under changing environmental conditions. The aim of this research was assessment of *P. nigra* viability in one of the best preserved riparian ecosystems in Europe, Special Nature Reserve "Gornje Podunavlje" (Upper Danube), Serbia. Additionally, the analysis of the genetic diversity was made to support the effective conservation in the future.

During our study, we have mapped 931 *P. nigra* trees, which were used for the assessment of present native population. Furthermore, we used 14 microsatellite markers to assess the genetic structure of this this population.

Viability assessment showed considerable occurrence of *P. nigra* in the research area, even though the results show fragmentation. *P. nigra* occurs mostly individually or in small groups of trees, and has a non-sustainable age structure due to insufficient or lacking regeneration. Despite the limited size of the studied population, the apparent overall genetic diversity was high (He = 0.759) and comparable to other known native populations of *P. nigra* along the Danube basin. However, the results also confirmed existence of recent bottleneck effect. Significantly positive and quite high F_{is} value (0.147) was noted, which may be ascribed to the "Wahlund effect" because of the population substructure that was revealed by the STRUCTURE analysis (K=2).

Although results say that coverage of native stands is not so promising, most of selected trees within our research assessed showed good viability with potential for natural reproduction However, the problem is that suitable areas for natural seedling establishment are scarce and with that gene flow is probably limited. The fragmentation of the area must be reduced and isolated stands must be interlinked as there is need to create larger non-fragmented areas.

Keywords: Black poplar, viability assessment, genetic diversity, SSR markers, SNR Upper Danube

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Introduction

Genetic diversity is of great importance in enabling a population to survive and reproduce under constant changing environmental conditions. A decrease in genetic variation has been correlated with the reduced adaptive flexibility, which threatens the ability of a species to survive via natural regeneration and has deleterious effects on the species fitness (Wang et al., 2011). Existence of large variations within a population increases chances that at least some individuals will be capable to adapt to new environmental conditions, allowing natural selection to result in adaptation (Ivetić et al., 2016). Therefore, the information on the genetic diversity or genetic structure in the remaining populations of endangered species is a prerequisite for the successful management and development of effective conservation strategies (Pospíšková & Šálková, 2006; Wang et al., 2011).

Black poplar (*Populus nigra* L.) is an important riparian pioneer species widely distributed across Europe, central Asia and northern Africa. It is one of the rarest and most endangered tree species in Western and Central Europe (Pospíšková & Šálková, 2006). It has been estimated that 99% of the riparian forests in Europe disappeared since the beginning of the 20th century (Lefèvre et al., 1998; Smulders et al., 2008), due to human activities. The main reasons are over-exploitation, frequent and broad use of hybrid poplars which may represent a great risk for genetic introgression of foreign germplasm into native *P. nigra* populations, and hydro-melioration activities that alters natural flow regime, which caused a lack of suitable sites for the natural regeneration (Čortan et al., 2016); all these take place also in the researched area. Recently, many subpopulations of black poplar have been identified as unit in strong age structure, where successful natural regeneration is absent due to inappropriate conditions (Rathmacher et al., 2010).

Like many countries in Europe, we lack basic information on the extent and special distribution of the remaining *P. nigra* population in Serbia, mostly because *P. nigra* is not separated in forest inventories. The many isolated trees or smaller groups are generally not included in the regular forest inventories. If they do occur sporadically in mixed stands, they will be identified as a group of other soft wood species, grouping *P. nigra* together with *P. alba* and *F. angustifolia*. Several research on genetic diversity assessment on Serbian *P. nigra* population have been done lately (Maksimović et al., 2014; Jelić et al., 2015; Čortan et al., 2016), but we do not have the complete picture of our research area, i.e. a clear picture which would cover both spatial and genetic distribution of selected population.

Hence, the aim of this research is to make viability assessment for the remnant population in the area of Special Nature Reserve "Gornje Podunavlje". Using microsatellite markers, genetic structure was also investigated so that appropriate and effective conservation measures could be prescribed in the future.

Materials and methods

Study area

The research was performed in Special Nature Reserve "Gornje Podunavlje" which is a unique and compact forest and marshland complex. It covers the alluvial plain and terrace of the left bank of the upper Danube basin in the Republic of Serbia, from the 1367th to 1433rd km of its course. The research area represents remnants of the former vast inundated parts of the Danube basin, covering the area of 19,648 ha without significant exposure, at an altitude between 82 and 87 m. Forest vegetation is primarily conditioned by floodwater or groundwater impacts of the Danube water level (Bobinac et al., 2010). There are lower areas of poplar and willow with annual flooding periods of 65 days and higher areas dominated by oak, ash, elm with the annual flooding periods of 30 days in vegetation period. Presence of black poplar native stands in this research area is rare and fragmentary, the natural regeneration of poplar is considerably reduced and the remaining trees are ageing.

Viability Assessment

As regular inventory data, we measured diameter and height of 931 *P. nigra* trees and took the coordinates of those trees with a GPS device and mapped them on the research area (Fig. 1). A tree diameter was measured by an electronic caliper at the height of 1.30 m above ground, while the height was measured by a clinometer. The diameter and height of the each selected black poplar trees were measured and grouped into the size classes. For the diameters, there were used 10 cm size classes, while for height 5 m size classes. Beside the assessment of vitality, health condition and natural regeneration potential assessments were done as well, so that we could establish present state of the studied population. For both assessments, we used scales from 1 to 4.

For the vitality and health assessment it was used condition system developed by the professional community, the Council of Tree and Landscape Appraisers (CTLA, 2000). The CTLA system (CTLA, 2000), is implemented in the various tree appraisal method and serve as a good tool for the valuation of plants (Cullen, 2007). It is a subjective assessment of the

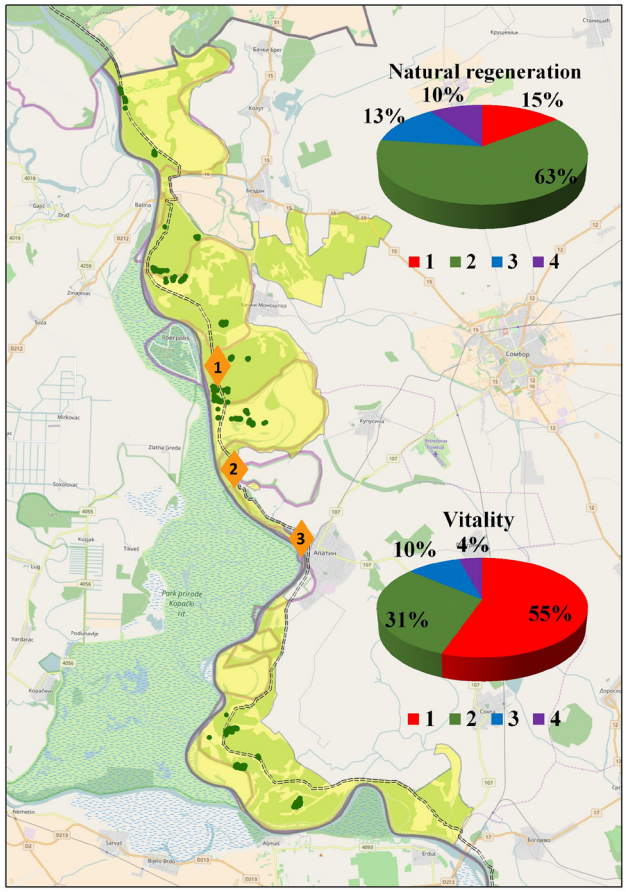

Fig. 1. Distribution of localities (1–3) and mapped black poplar trees along SNR "Gornje Podunavlje", along with the graphs representing assessment grades (1–4) for vitality and natural regeneration

tree's structural integrity and health at the time of appraisal, given by trained and experienced professional who has been on site. According to Bond (2014), the system breaks condition into five factors (rated from 1–4) and combines them to obtain a final condition rating. These five factors are: roots, trunk, scaffold branches, small branches and twigs and foliage.

Within vitality and health conditions, the grade 1 was for those individuals that are completely functional and healthy, grade 2 – functional individual, average vitality, grade 3 – limited functionality and vitality under average, not good health condition, and grade 4 – not functional individuals.

Regarding the natural regeneration ability assessment, grade 1 was for those individuals with great regeneration potential, grade 2 – was for those individuals with decent regeneration potential, grade 3 – was for those individuals with poor regeneration potential, while grade 4 for individuals that are not capable for regeneration. Grades 2 and 3 were between the previous limited values. This assessment is related to the probability of success using natural regeneration, and it is determined by the evaluation of sprouting potential of the overstory trees. However, during the evaluation several other factors were assessed including: density, distribution, dimensions, and condition of any advanced regeneration (seedlings and saplings already in the understory) as well as site quality.

Genetic analysis

Leaves from 30 adult *P. nigra* individuals, identified based on morphology, were collected for genetic analyses. Selected individuals have been grouped in tree randomly selected localities, by 10 individuals, separated up to 10 km from each other (Fig. 1). Each individual was randomly selected and separated at least 100 m from the next one to avoid clonal structure due to root suckers to the largest possible extent (Wei et al., 2013; Čortan et al., 2016). All selected individuals have been evaluated as the one of good vitality (grade 1 and 2). The samples were collected in October, 2013; they were dried and stored in plastic grip seal bags with silica gel prior to DNA isolation.

DNA extraction and marker analysis

DNA was extracted according to the protocol of Dumolin et al. (1995), while the polymerase chain reaction (PCR) was performed according to Pakull et al. (2009), in a total volume of 25 μl containing 80 ng of template DNA (details in Čortan et al., 2016). Annealing temperatures were in the range of 50–65°C, depending on used primers (Supplementary material – Table 1).

In total, 14 nuclear microsatellite (nSSR) loci were used (PMGC_14, PMGC_108, PMGC_2020, PMGC_2163, PMGC_2550, PMGC_2607, PMGC_2679, GCPM_354, WPMS_09, WPMS_14, WPMS_16, WPMS_17, WPMS_18, WPMS_20). Those with PMGC and GCPM prefix were selected from International *Populus* Genome Consortium IPGR SSR resource (http://www.ornl.gov/sci/ipgc/ssr_resource.htm); the one with the WPMS prefix were developed by the Center for Plant Breeding and Reproduction Research (van der Schoot et al., 2000; Smulders et al., 2001).

The PCR products were separated using the automatic sequencing unit ALFexpress II (GE Healthcare) under same conditions as in Čortan et al. (2016): running time 105 – 180 min, short gel plate with 6% polyacrylamide gel, running temperature 55°C and voltage 1,500 V. Samples were prepared according to Pakull et al. (2009). Data analysis was carried out using the Fragment Analyser software (version 1.03.01, GE Healthcare).

Population genetics analysis

The following genetic diversity parameters were determined for each used locus: number of alleles (N_a) and mean effective number of alleles (N_e), observed (H_o) and expected (H_e) heterozygosity (Nei, 1973), inbreeding coefficient (F_{is}) (Weir & Cockerham, 1984). We also calculated Shannon's indices (SI) to characterize genetic variation within the studied population. These parameters have been performed using the Arlequin software version 3.5.1.2 (Excoffier & Lischer, 2010) and Fstat (Goudet, 1995). Test for the bottleneck (Garza-Williamson Index – M-ratio, Garza & Williamson, 2001) and Principal Coordinates Analysis (PCoA) were performed with the help of GenAlEx version 6.501 software (Peakall & Smouse, 2006). We have also used the software package STRUCTURE v2.3.4 (Pritchard et al., 2000) to assess population structure of *P. nigra* in studied area. This program applies a model-based clustering algorithm that implements the Markov chain Monte Carlo (MCMC) algorithm and a Bayesian framework. The algorithm identifies subgroups with distinctive allele frequencies and places individuals into *K* clusters using its estimated membership probability (*Q*). Runs were performed with a burn-in time of 10,000 repetition and a MCMC of 100,000 iterations using the admixture model, and using K (possible number of clusters) from 2–12. Afterwards it was used an *ad hoc* procedure by Evanno et al. (2005) based on the second-order rate of change in the log probability (ΔK) of data between successive *K* values, performed in STRUCTURE HARVESTER program (Earl & von Holdt, 2012).

Results

Viability Assessment

Using CTLA system for evaluating tree health and vitality we have mapped and assessed 931 tree from the native black poplar. The results (Supplementary material – Fig. 1) shows that most of the black poplar individuals (55%) got the grade 1, showing great functionality and health. There were many trees that showed average vitality (31%, grade 2). The proportion of trees with limited vitality was noticeably lower at 10% (grade 3) and only 4% of selected trees are completely without functionality (grade 1). These results as whole gave a good understanding to vitality, and showed that the most of the selected trees are functional and healthy.

Regardless of inappropriate conditions, old age structure, solid vitality and limited natural stands of the present population within the studied area, the successful regeneration still exists, but is rather sporadic and it is mostly vegetative. The results (Fig. 1 and Supplementary material – Fig. 2) of natural regeneration potential of selected black poplar individuals, at the first glance shows clearly that the majority of individuals have grade 2 (63%), which are the one with decent regeneration potential. Great regeneration potential (grade 1) showed 15% of total selected trees, and 13% showed poor regeneration potential (grade 3). Individuals that are not capable for regeneration (grade 4) accounted only 10%. This shows that significant number of black poplar trees is not capable for regeneration at all. However, all this regeneration is primarily vegetative.

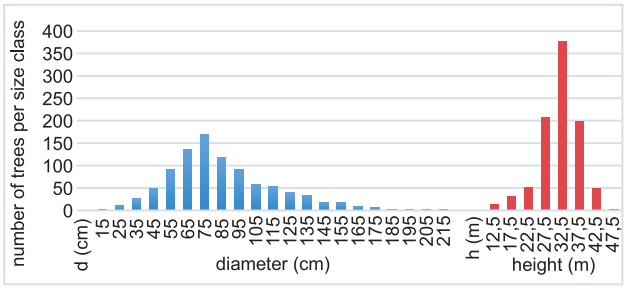

Fig. 2. Black poplar tree distribution in size classes by diameter and height

Looking at the maps we could notice that there is no specific distribution pattern of individuals with different level of vitality (Supplementary material – Fig. 1) and natural regeneration potential (Supplementary material – Fig. 2); they are all randomly scattered across the area. Therefore, the individuals with the lowest or highest level of vitality and individuals capable or incapable for regeneration are not clustered around a particular zone within the research area.

Fig. 2 shows that the most of selected trees have diameter and height in higher size classes. The majority of selected trees belonged to size classes that comprised diameters between 51 and 100 cm. For the height measurements, the most of the trees are between 25.1 and 40 m. Although, there is no direct measurement for age, dominant diameter and height distribution in the size classes indicates that most of trees are mature or overmatured.

Genetic diversity and population structure

Microsatellite profiles of the analysed individuals from black poplar population in the researched area showed, by using the test of probability of identity (PI) that there are no identical genotypes within 30 selected individuals. While the LRM estimator (Lynch & Ritland, 1999) with mean value of –0.017 (ranging from –0.122 to 0.152) showed that there is no relatedness between these individuals.

Table 1. Genetic diversity parameters determined for studied population of *P. nigra*

Locus	N	N_a	N_e	SI	H_o	H_e	Fis	G-W index
PMGC_14	30	6	4.523	1.610	0.700	0.779	0.101	0.857
PMGC_2020	30	8	3.005	1.428	0.500	0.667	0.251	0.667
PMGC_2163	30	15	11.688	2.553	0.867	0.914	0.052	0.421
PMGC_2550	30	5	2.442	1.133	**0.633**	**0.591**	**–0.072**	0.882
PMGC_2607	30	13	8.257	2.314	0.567	0.879	0.355	0.455
PMGC_2679	30	6	4.286	1.578	0.567	0.767	0.261	0.419
PMGC_108	30	8	2.769	1.446	0.400	0.639	0.374	0.857
GCPM_354	30	7	3.822	1.548	0.433	0.738	0.413	1.000
WPMS_9	30	12	10.526	2.409	0.733	0.905	0.190	0.923
WPMS_14	30	11	5.751	2.029	0.800	0.826	0.032	0.688
WPMS_16	30	6	3.152	1.373	0.600	0.683	0.121	0.857
WPMS_17	30	5	3.087	1.241	**0.733**	**0.676**	**–0.085**	0.500
WPMS_18	30	8	4.306	1.676	0.767	0.768	0.001	0.800
WPMS_20	30	9	4.737	1.844	0.733	0.789	0.070	0.818
Mean	30	8.5	5.168	1.727	0.645	0.759	0.147	0.725
SE	0	0.837	0.782	0.118	0.037	0.027	0.044	0.193

N = Number of samples; N_a = No. of Different Alleles; N_e = No. of Effective Alleles; SI = Shannon's Information Index; H_o = Observed Heterozygosity; H_e = Expected Heterozygosity; F_{is} = Fixation Index; G-W index - Garza-Williamson index.

The analysis of 14 nSSR loci (Table 1) showed that studied population had in total 119 different alleles, on average 8.5 alleles per each locus. Number of effective alleles per locus (N_e) ranged from 2.442 (PMGC_2550) to 11.680 (PMGC_2163). The observed heterozygosity (H_o) per locus ranged from 0.400 (PMGC_108) to 0.867 (PMGC_2163), while expected heterozygosity (H_e) per locus values ranged from 0.600 (PMGC_2550) to 0.911 (PMGC_2163). The expected heterozygosity for each locus was generally higher then observed heterozygosity. However only two loci, PMGC_2550 (F_{is} = –0.072) and WPMS_17 (F_{is} = –0.085), showed a negative F_{is} values indicating heterozygote excess (outbreeding); for most loci F_{is} values were significantly positive indicating homozygote excess (inbreeding). The overall value of F_{is} was 0.147 and statistically significant, in the range from –0.085 (WPMS_17) to 0.413 (GCPM_354) ($P < 0.001$). Shannon's information index ranged from 1.133 (PMGC_2550) to 2.553 (PMGC_2163), with an average of 1.727 alleles (Table 1).

Moreover, we further investigated a genetic bottleneck using the G–W index, also called M-ratio, which represents the ratio of number of alleles to range in allele size. The analysis of bottleneck indicated the overall mean value of G–W index about 0.7, which is specific for population that has gone through a recent population size reduction. This was supported by results of Wilcoxon signed-rank test implemented in Bottleneck software, showing distribution of alleles with a shifted mode ($P = 0.04688$) which is expected in a population that has experienced a recent reduction in size (population bottleneck).

The Hardy-Weinberg equilibrium (HWE) test (Table 2), indicates obvious that the probability of Chi – square values (taking into account the degree of freedom – DF) for loci PMGC_14, PMGC_2163, PMGC_2550, WPMS_16, WPMS_17, WPMS_18 were higher than 0.05 (in the range $0.05 < P < 1.0$), thus results for this set of loci were not statistically significant. Considering that the probability of Chi – square values for PMGC_2020, PMGC_2607, PMGC_2679, PMGC_108, GCPM_354, WPMS_9, WPMS_14, WPMS_20 were less than 0.05 (in the range $0 < P < 0.05$), it shows that the results for this set of loci were statistically significant, showing significant deviation from Hardy-Weinberg equilibrium.

Afterwards, we assessed the structure of differentiation using a PCoA analysis of all individual trees (Fig. 3), without prior classification information. In the plot, it could be seen that our individuals are separated by 3rd axis in the two groups, showing

Table 2. Significance deviation test from Hardy-Weinberg equilibrium (HWE) per each locus (ns=not significant, * P<0.05, ** P<0.01, *** P<0.001)

Locus	DF	ChiSq	Prob	Signif
PMGC_14	15	13.814	0.540	ns
PMGC_2020	28	56.700	0.001	**
PMGC_2163	105	104.288	0.501	ns
PMGC_2550	10	2.720	0.987	ns
PMGC_2607	78	133.215	0.000	***
PMGC_2679	15	29.949	0.012	*
PMGC_108	28	60.288	0.000	***
GCPM_354	21	54.581	0.000	***
WPMS_9	66	128.092	0.000	***
WPMS_14	55	96.127	0.001	***
WPMS_16	15	23.334	0.077	ns
WPMS_17	10	6.306	0.789	ns
WPMS_18	28	40.264	0.063	ns
WPMS_20	36	51.853	0.042	*

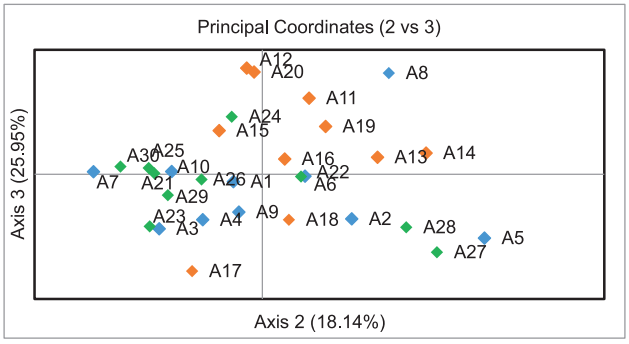

Fig. 3. Principal Coordinate Analysis (PCoA) of native *P. nigra* population based on genetic distances, explained by 2nd and 3rd axis. Different colours represent different localities

cumulative percent variation of 25.95%. The structure of genetic differentiation within studied population was consistent with the analysis revealed by STRUCTURE, separating individuals into two clusters (Fig. 4). Specifically, from PCoA and STRUCTURE graphs one could see that in this separated group there are mostly individuals numbered from 11–20 representing second locality, with exception of number 8 and 24, each from other locality, while other two localities are mixed up even though they are not close as they are with second locality (Fig. 1).

Discussion

Viability Assessment

Inventories of natural resources, assessment of their viability and natural regeneration potential are the first step in providing insight of the level of threats to the given species. Many countries in Europe had only rough data about *P. nigra* occurrence available, but the actual situation was far from the assessed, considering that many trees are not included in the forest inventories or they have been group in "poplars" group which does not allow for detailed identification of the species (Lefèvre et al., 1998). Inventories specifically dedicated to *P. nigra* are still exceptional in many parts of Europe, but thanks to the EUFORGEN *P. nigra* Network many member countries made an inventory and assessment of native stands, but this network did not cover most of Balkan Peninsula. Up till now specifically dedicated assessment study of *P. nigra* in the research area of SNR "Gornje Podunavlje", and as well as in whole Serbia, is yet to be made.

According to Banković et al. (2009) in the total growing stock of Serbia the share of autochthonous poplar forests accounts with only 0.5–1.0%, and therefore they are considered as a rare species. Present situation is a consequence of both dramatically changed habitat conditions of remaining riparian forests and of hybrid poplars cultivation on the native habitats of *P. nigra*. (Heinze, 1997). Herpka (1979) mentioned that first poplar hybrids were massively introduced in the research area at the end of 19th century, from Western Europe, when the significance of the native riparian species began to decrease rapidly. If we look in total research area, it has been mostly covered with selected, highly-productive cultivars of poplars from section *Aigeiros* Duby (Ivanišević et al., 2009). These hybrid plantations cover the area of about 5,000 ha, which is about 25% of the total research area. However, fragmentary native stands of black poplar within the research area cover about 315 ha, which is about 1.6% of the total area (or 0.6% in total volume), and they mostly represent overmatured trees. Furthermore Herpka (1979) mentioned that water regulation works started in 19th century, when the natural dynamics of the ecosystem (periodic flooding) and the lateral movement of the river bed have been dramatically affected and the habitat conditions started to change, so that the area available for *P. nigra* natural regeneration have been reduced and softwood species have been progressively replaced by hardwood species (e.g. *Fraxinus*, *Acer*, *Ulmus*, *Quercus*) (Imbert & Lefèvre, 2003). These remaining fragments of black poplar stand take on characteristics of the habitat islands in the landscape, which are highly sensitive to changing conditions, such as those caused by climate change, habitat fragmentation or land-use change and urbanization (Schmied, 2002).

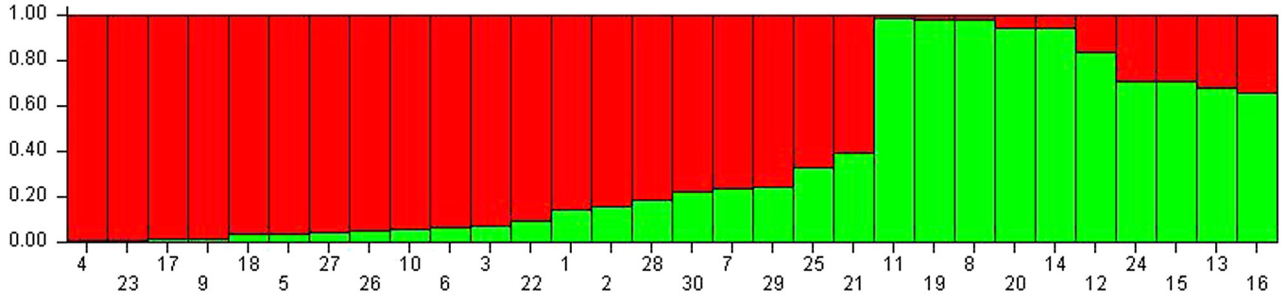

Fig. 4. Population structure analysis of native *P.nigra* population, with K = 2

Fig. 5. Group of trees in its natural habitat (photo by Čortan D. 2017)

During our study, we have mapped 931 trees, which were used for the condition assessment of the present native population, the most of selected trees has diameter 71–80 cm, and height 30.1–35 m (Fig. 2). According to the management plans, most of these trees are over 36 years old. However, considering that many of selected trees were not part of previous inventory, and according to the present state and dimension distribution of selected individuals within mixed stands, it is estimated that they could be older than 36 years. Although there is no direct measurement of the age we could firmly say that these individuals are mature or overmatured, with considerable number of them being basically dead (almost 15% of assessed individuals, graded with grades 3 and 4). The obtained results show fragmentation within the research area, where black poplar occurs individually or within smaller groups of trees (Fig. 5).

According to the used system of vitality assessment, most of individuals had grade 1 (56%) and 2 (31%), which implies that those are functional individual with good vitality. However, when it comes to vulnerability assessment, we have to be aware that a certain number of assessed individual was not in good health condition or they were dead. Natural regeneration assessment showed 63% of these individuals had a grade 2, meaning that these individuals are still showing good regeneration potential. Furthermore, 15% of individuals had a grade 1, showing excellent regeneration potential. However, we should bear in mind, that this regeneration potential is primarily vegetative, considering that sites for generative regeneration are rare. Furthermore, we did not noticed any specific patterns in the map regarding individual's grade distribution, even though microenvironment conditions of selected individual have not been uniform. Through this assessment, we did not notice any specific environmental conditions which could influenced on level of vitality or on capability of regeneration of assessed individuals, but this requires more detailed investigation regarding environmental factors.

Regardless of inappropriate environmental conditions for natural regeneration, old age structure, solid vitality and limited natural stands of the present population within the studied area, the successful regeneration still exists, but is rather sporadic. Considering relatively good vitality of most of remaining trees, vegetative regeneration is possible and widespread where the natural habitat allows for it. However, it can be noticed that this regeneration is only possible within area where game does not have an access; during the winter period the young sprouts are attractive food for various game species. As far as generative regeneration is concerned, *P. nigra* is known as a species which

colonises an open area on fresh alluvial soil through seed, and requires specific water-soil conditions for germination. Considering that there is no appropriate area for seed germination due to river system regulation done in the past and significantly changed hydrological regime, generative regeneration is really seldom and occurs only next to the river banks.

However, in comparison to European populations, it is considered that gene pool of the remaining P. nigra populations is still well preserved in Balkan area (Kajba et al., 2015). Although the species may demonstrate good viability locally and highly successful regeneration, as a case in our research area, some European regions have witnessed significant reduction in populations or the complete disappearance of black poplar (Vanden Broeck, 2003; Toplu, 2005), such as Belgium, Netherlands, Greece, United Kingdom (Lefèvre et al., 1998). According EUFORGEN Black poplar network's reports (Turok et al., 1999; Koskela et al., 2004), in Romania there is 80,000 ha of native poplar forests, in Russia there is about 100,000 ha of black poplar stands, in Germany along the river Eder 800 individuals and in upper Rhine 1,124 individuals have been registered and in Switzerland less than 1,000 individuals are estimated as pure *P. nigra*. Cooper et al. (2002) estimated that there are around 7,000 trees in Britain, and the similar situation is in many other parts of Europe. However, in comparison to other European countries our results show that presence of *P. nigra* in the research area is still considerable. We have selected only 931 individuals for assessment and there is many more in this area covering almost 20,000 ha. Balkan Peninsula is already known as potential refugium of many plant species, and it is supposed as one of refugium for *P. nigra* (Cottrell et al., 2005), which explains still satisfying number of this endangered species in this area.

Considering that *P. nigra* lacks high, direct commercial use in forest management in Serbia, as well as in entire Europe, its absence from management plans makes sense and as well as its absence from the inventory. Even though it is not an economically interesting species, it is of great environmental importance for endangered riparian ecosystems, having in mind that it plays a central role in the development of riparian ecosystems contributing to the natural control of flooding and water quality, and serving as a natural corridor that facilitates gene flow for many riparian species (Storme et al., 2004; Naiman et al., 2005; Jelić et al., 2015).

Genetic diversity and population structure

nSSR are ideal markers for the assessment of genetic diversity and population structure in plant species since they are co-dominant and highly informative (Blair et al., 2010). Studied *P. nigra* population shows a considerable genetic diversity within the studied area (H_e=0.759). Even though there is limited size of the population, a level of genetic diversity is consistent with other European populations on local and global scale (H_e=0.82, Pospíšková & Šálková, 2006; H_e=0.73, Imbert & Lefèvre, 2003; H_e=0.73, Ratchmacher et al., 2010; H_e=0.822, Maksimović et al., 2014; H_e=0.811, Jelić et al., 2015; H_e=0.808, Čortan et al., 2016; H_e=0.792; Lewandowski & Litkowiec, 2017), with consideration on the differences in sample sizes and different number of loci used. The values of H_o and H_e were similar among analyzed loci, showing in general higher values of H_e and confirming positive value of F_{is}. The positive F_{is} indicates excess of homozygotes in the studied population (overall F_{is}=0.147). However, only when all studied loci show equally high F_{is} values, in our case ranging from –0.085 to 0.413 and been positive in 12 loci out of 14, it could be suggested possible existence of mating among close relatives which could lower the heterozygosity of studied population. Due to the presence of significantly positive and quite high F_{is} value for dioecious species as *P. nigra* and presence of substructure (K=2) in the studied population an alternative explanation for overall heterozygote deficiency might be the existence of "Wahlund effect".

However, comparing number of alleles with the other populations using the same loci we used in our analysis, we can notice twice lower values, which we could assume that is definitely in correlation with number of samples, considering that other studies had more populations and samples. Only the research performed by Maksimović et al. (2014) and Pospíšková and Šálková (2006) showed similar number of alleles as in our population, having in mind that they studied one population one with 30 and another with 60 individuals. This was also noticed in the research performed by Pospíšková and Šálková (2006), stating that this assumption is supported by some population geneticists (Mousadik & Petit, 1996; Comps et al., 2001; Widmer & Lexer, 2001) saying that most relevant criteria for the diversity measurement, especially in the context of genetic conservation, should be the number of alleles per locus (as allelic richness) rather than gene diversity (known as the expected heterozygosity).

The structure of genetic differentiation within studied population show consistency within PCoA and STRUCTURE results, showing two clusters, but there were not completely consistent with geographical distribution of studied localities. The middle locality (number 2) with two individuals from other locations showed separation, while other two localities have mixed up genes with two additional individuals from middle location. Even though we have noticed

fragmentation within our studied area, genetic differentiation results could be explained with existence of gene flow in the research area. Besides a little grouping within individuals from second locality, we still have wide spread genes along our population. Both the seed and pollen of *P. nigra* are widely dispersed primarily with wind (Imbert & Lefèvre, 2003; Rathmacher et al., 2010), which provides logical explanation for structure observed in our study. Moreover, it is possible that the primary genetic structure of studied population could have been distorted by human activity in the past (Lewandowski & Litkowiec, 2017).

Furthermore, the mean value of G–W index was about 0.7, representing recent reduction in population size, the most loci showed values over 0.8, representing no reduction history. However, five loci showed values lower than 0.7 indicating recent size reduction, while only one loci (GCPM_354) was even lower, lower than 0.43, which indicates significant population decline in the past (Garza & Williamson, 2001; Tzika et al., 2008). The mean value of G–W index was confirmed with alleles shifted mode distribution, confirming existence of recent size reduction within studied population. This could be also confirmed with already mentioned twice lower number of alleles per locus in our study than in other studies. Conservation biologists widely agree that population bottleneck should be avoided in threatened species because they can increase rate of inbreeding, loss of genetic variation and fixation of mildly deleterious alleles, and thereby reduce adaptive potential of the species and increase the probability of their extinction (Cornuet & Luikart, 1996; Peery et al., 2012). However, even though we assumed that the population included in our study has experienced recent demographic decline, followed with existence of week inbreeding within population, we still have considerable diversity which has to be preserved urgently.

Selected measures and achievements

One should bear in mind that *P. nigra* as a pioneer species requires a particularly high level of genetic diversity, if its survival is to be guaranteed by retaining its capability to adapt to the changes associated with a dynamic river system which is its natural environment (Booy et al., 2000). The maintenance and conservation of the existing genetic diversity provides the potential for tree species to adapt to long-term environmental changes (Hedrick, 2006). Conservation measures should primarily focus on the maintenance of this, still diverse population with good regeneration potential, which can be considered as a source population for further restoration projects. Sufficient natural regeneration is urgently needed to compensate for the loss of genetic diversity caused by population obsolescence (Rathmacher et al., 2010). Lefèvre et al. (2002) recommends identifying and preserving the local and regional gene pools whenever possible and to use reproductive material locally. This process must be integrated into forest policies on the local and the state level. Besides, the fragmentation of the area must be reduced by adapted measures and isolated stands must be interlinked as there is a need to create larger non-fragmented areas. As already said priority should be put on avoiding the loss of suitable habitats for *P. nigra* and preventing the emerging discontinuities in the riparian forest (Imbert & Lefèvre, 2003).

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