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CHARACTERIZATION OF SERBIAN SPRUCE VARIABILITY APPLYING ISOENZYME MARKERS

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ABSTRACT

The Serbian spruce seedling seed orchard at Godovik represents a base for production improvement of selected seed material from this species, which can be used as initial material for extension of its range. This research paper covers the five phenogroups which confirmed the existence of the characteristic traits in the second known generation. To define the genetic distance between the studied phenogroups, to assess intraspecific variability and inbreeding, 16 loci in 12 isoenzyme systems were used. It is recommended to cross the genotypes of the phenogroups "B" and "D" as genetically most similar ones, which would possibly result in a new variety characterised by double-topped semi-dwarf specimens. Along with this model, all other hybridisations among phenogroups "B", "D", "F" and "C" are also supposed to be successful. The establishment of an adequate hybridisation model within the above mentioned phenogroups, orientated to unification of their positive ornamental, productive and ecological characteristics, is the next phase of enhancement of the species' genetic structure and its utilisation.

Keywords: Serbian spruce, intraspecific variability, isoenzymes

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Introduction

The Serbian spruce *(Picea omorika Pančić/Purkyne)* is a Balkan Peninsula endemic and Tertiary relic. Today, the Serbian spruce can be found as a native species only on the Balkans, i.e. in the narrow area along the middle and the upper course of the river Drina, at the border of eastern Bosnia and western Serbia. In Bosnia and Herzegovina, it is presented at 13 localities (6, 7), and in Serbia there are twenty small isolated populations or tree groups. According to the data of *The World Conservation Monitoring Centre,* on the area of 60 ha in the National Park Tara, there are only about one thousand Serbian spruce trees in mixed stands with spruce and beech. The Serbian spruce was an object of research of more than 700 scientific papers of different subjects: general data on the species, origin of species and its taxonomic relation to other species, Serbian spruce sites and distribution, technical characteristics, anatomic structure and morphological description of macro preparations of Serbian spruce wood. The subjects also included Serbian spruce diseases and pests, variability from the aspect of morphological and physiological characters, pollen properties, Serbian spruce resistance to air pollution, etc.

The studies on Serbian spruce genetic structure by molecular markers are few. Maternal inheritance of mitochondrial DNA in interspecific crosses of *Picea glauca* (white spruce) and *Picea omorika* was shown by David and Keathley (5). The genetic structure of both a Serbian spruce natural population at Stolac (near Višegrad in Bosnia and Herzegovina) and an artificially established plantation in the Arboretum Punkaharju in Finland has been studied by parallel analyses of isoenzyme markers and quantitative traits (14). The results of the above mentioned studies indicated extremely high level of genetic variation in both populations. The genetic variation of five natural Serbian spruce populations from Bosnia and Herzegovina has been studied by analysis of 12 enzyme systems and 16 gene-loci (1). The results of these studies showed significant differences in the genetic structure among the populations from the area of Višegrad. It was concluded that the seeds and cones should be separately declared before sending them to the market, which had not been practiced, as the declaration referred only to the Višegrad provenance. The genetic variation of 12 natural populations and one plantation from Bosnia and Herzegovina was studied by an analysis of 16 isoenzyme loci (2). Most of the populations had a significant number of heterozygotes, which pointed to a potent selection against inbreeding individuals. Allele polymorphism of *nad1* gene of the Serbian spruce mitochondrial genome was analyzed and it was concluded that there was a degree of genetic control on the phenotype expression in different Serbian spruce phenogroups (16).

Materials and Methods

The research was performed on the material collected from the Serbian spruce seedling seed orchard in the village Godovik, Municipality Požega, Republic of Serbia (25). In the experiments with genetically defined material (clones, provenances, half-sib lines), the degree of genetic control of phenotypic traits can be determined directly, by the phenotype differentiation of the demes (small subpopulations) in a well-planned experiment, if all the material is exposed to identical environmental conditions and treatments (10). The planting pattern in the seedling seed orchard at Godovik satisfies these

criteria, which shows that the genetic research within this *ex situ* conservation unit is justified.

A great part of the genetic variation and the potentials of Serbian spruce natural populations have been incorporated in three plantations situated in Western Serbia. In this way, research and future utilisation were much more available.

The intensive research of the plantations started by the classification of trees into phenogroups which were considered to be significant for forestry and horticulture (11):

- **phenogroup "A"** - variety ***"borealis"***, branching very similar to Norway spruce, wide crown;
- **phenogroup "B"** - variety ***"semidichotomy"***, without visible biotic and abiotic causes, the spontaneous dichotomy – "false dichotomy";
- **phenogroup "C"** - variety ***"serbica"***, branching type and habit characteristic of Serbian spruce, narrow-pyramidal crown;
- **phenogroup "D"** - variety ***"nana"***, semi-dwarf, maximal height up to 1.80 m;
- **phenogroup "E"** - dwarf, maximal height up to 0.7 m;
- **phenogroup "F"** - type ***"argentea"***, needles on current-year and second-year branchlets point upwards giving silvery appearance to the crown;
- **phenogroup "G"** - type ***"viminalis"***, current-year and second-year branchlets pendulous 30 to 50 cm down the branches.

The Godovik seed orchard represents the second known generation of individuals whose phenotypic traits correspond to the definition of some of the phenogroups. It was only the inheritance of traits characterising the phenogroup "E" that was not confirmed in the second generation.

In order to define the genetic distance between the studied phenogroups and to assess the population and intraspecific variability and inbreeding, isoenzymes were applied as codominant markers.

The research presented in this paper covers the five phenogroups ("A", "B", "C", "D" and "F") which confirmed the existence of the characteristic traits in the second known generation.

For genotyping, 16 loci in 12 enzyme systems were used: ***Per, Gdh, Pgm, Sdh, Got, Ndh, Mdh, Fest, Dia, Pgi, Idh*** and ***Lap*** (**Table 1**). Enzymes were extracted from dormant winter buds using a 0.1 M Tris-HCl buffer pH 7.3 with the addition of 1% PVP 40, PVP 80, PVP 360, EDTA II, Tween-80, PEG, and 2-mercaptoethanol, 0.025% DTT, and 0.5 M Na-ascorbate. The homogenates were subjected to horizontal starch-gel electrophoresis using 12% (w/v) gels. The protocols for the electrophoresis and staining essentially followed (4). The interpretation of zymograms followed the studies done by Ballian et al. (1). The genotypic and allelic frequencies were calculated and the genetic variation within phenogroupes was characterized by the total number of alleles, the proportion of polymorphic loci (because of the relatively small sample sizes, each locus, where more than one allele was found, has been considered as polymorphic), and the observed and expected heterozygosities (17). The program BIOSYS-1 (21) was used for all calculations. Departures from panmixia within phenogroupes were assessed by fixation indices (9). To assess the trends of genetic differentiation, we calculated genetic distances (following unbiased estimates) (17) between the phenogroupes.

Results and Discussion

Revealing, selection and defining of different phenogroups within the above mentioned plantations, which preceded the seed orchard at Godovik, was a significant step in the study of Serbian spruce intraspecific variation, which had previously been characterised as exceptionally low. The phenotypic determination of different intra-specific varieties and types showed opposite results and it opened numerous questions on the degree of genetic diversity, genetic control of the phenotypic expression, the degree of inheritance of characteristic traits and the newly revealed potential for further breeding of these species (12, 13).

By selection of superior genotypes within each phenogroup in plantations, by seed collection and by establishment of the Godovik orchard, the process towards the improvement of the species' genetic structure continued. As these characteristic traits were observed also in the orchard, the hypothesis on their inheritance was confirmed.

Among the 15 detected gene loci in different Serbian spruce phenogroups, eight gene loci were monomorphic (***Shd-B***; ***Got-A, Got -B, Got -C***; ***Ndh-B***; ***Mdh-A***, ***Mdh -C***; ***Idh-B***) and seven gene loci were polymorphic (***Per-A***; ***Gdh-A***; ***Pgm-A***; ***Mdh-B***; ***Fest-A***; ***Dia-C***; ***Pgi-B***) (**Table 1**).

A considerably lower number of polymorphic gene loci was observed in the previous research of enzyme systems of Serbian spruce from natural populations (1, 2). The polymorphic loci ***Pgm-A***, ***Fest-A***, ***Gdh-A*** and ***Mdh-B*** showed polymorphism in several previous analyses (1, 2, 14). The locus ***Pgi-B*** was characterised as polymorphic by Kuittinen et al. (14), but it exhibited monomorphism in the later research (1). The loci ***Per-A*** and ***Dia-C*** exhibited monomorphism in previous researches, but now they were determined as polymorphic.

Most polymorphic loci are diallelic, except for the locus ***Per-A***, which was threeallelic in phenogroups "A" and "F", and the locus ***Pgm-A***, whose three-allelism was observed in phenogroups "A", "B" and "C". The highest percentage of polymorphic loci was observed in phenogroups "C" and "D" (53.85), while in phenogroups "A", "B" and "F" it was somewhat lower (46.15). Such results only confirm the well-known fact on the exceptionally variable and barely explainable genetic structure of the rare, endemic and relic species, such as the Serbian spruce.

The total number of alleles varies insignificantly among phenogroups, but it generally shows a higher value compared to the previous research of Serbian spruce natural populations

TABLE 1

Enzyme systems, E. C. reference number, abbreviations, number of gene loci and number of found allels

Enzyme system	E.C. number	Abbreviation	Genlocus	Number of alleles
Peroxidase	1.11.1.7	*Per*	Per - A	3
Glutamatdehydrogenase	1.4.1.2	*Gdh*	Gdh - A	2
Phosphoglucomutase	2.7.5.1	*Pgm*	Pgm - A	3
Shikimic acid dehidrogenase	1.1.1.25	*Sdh*	Sdh - B	1
Glutamat oxalacetat transminase	2.6.1.1	*Got*	Got - A, -B, -C	1,1,1
NADH - Dehydrogenase	1.6.99.3	*Ndh*	Ndh - B	1
Malatdehydrogenase	1.1.1.37	*Mdh*	Mdh - A, -B, -C	1,2,1
Fluorescent esterase	3.1.1.1.	*Fest*	Fest - A	2
Diaphorase	1.6.4.3	*Dia*	Dia - C	2
Phosphoglucose isomerase	5.3.1.9	*Pgi*	Pgi - B	2
Isocitrat dehydrogenase	1.1.1.42	*Idh*	Idh - B	1
Leucin amino peptidase	3.4.11.1	*Lap*	-	-
Total **12**			**15**	**23**

TABLE 2

Allelic frequencies in Serbian spruce phenogroupes

Enzyme system	Genlocus	Phenogroupe				
		A	B	C	D	F
Peroxidase	Per - A1	0.143	0.000	0.000	0.000	0.033
	Per - A2	0.821	1.000	0.800	0.933	0.933
	Per - A3	0.036	0.000	0.000	0.000	0.033
Glutamatdehydrogenase	Gdh - A1	0.663	0.400	0.400	0.367	0.300
	Gdh - A2	0.779	0.600	0.600	0.633	0.700
Phosphoglucomutase	Pgm - A1	0.933	0.933	0.867	0.967	0.884
	Pgm - A2	0.077	0.033	0.067	0.033	0.117
	Pgm - A3	0.012	0.033	0.067	0.000	0.000
Shikimic acid dehidrogenase	Sdh - B1	1.000	1.000	1.000	1.000	1.000
Glutamat oxalacetat transminase	Got - A1	1.000	1.000	1.000	1.000	1.000
	Got - B1	1.000	1.000	1.000	1.000	1.000
	Got - C1	1.000	1.000	1.000	1.000	1.000
NADH - Dehydrogenase	Ndh - B1	1.000	1.000	1.000	1.000	1.000
Malatdehydrogenase	Mdh - A1	1.000	1.000	1.000	1.000	1.000
	Mdh - B1	0.520	0.700	0.567	0.533	0.734
	Mdh - B2	0.480	0.300	0.433	0.467	0.267
	Mdh - C1	1.000	1.000	1.000	1.000	1.000
Fluorescent esterase	Fest - A1	0.183	0.467	0.133	0.167	0.317
	Fest - A2	0.817	0.533	0.867	0.833	0.684
Diaphorase	Dia - C1	0.048	0.033	0.133	0.067	0.500
	Dia - C2	0.952	0.967	0.867	0.933	0.950
Phosphoglucose isomerase	Pgi - B1	0.865	0.733	0.933	0.833	0.950
	Pgi - B2	0.135	0.267	0.067	0.167	0.050
Isocitrat dehydrogenase	Idh - B1	1.000	1.000	1.000	1.000	1.000
Leucin amino peptidase	-	-	-	-	-	-

TABLE 3

Genetic variability

Phenogroupe	Total number of alleles	Number of alleles per locus	Percent of polimorphic loci	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Fixation index (Fst)
A	21	1.62	46.15	0.143	0.155	0.078
B	20	1.54	46.15	0.152	0.146	-0.041
C	21	1.62	53.85	0.167	0.156	-0.071
D	20	1.54	53.85	0.181	0.135	-0.341
F	20	1.54	46.15	0.152	0.136	-0.118

in Bosnia, which still attains only a half of the number of alleles determined in the Norway spruce (2). The number of alleles in Serbian spruce from the Godovik seed orchard was closest to the value determined by Ballian et al. (2), in the artificially established plantation Kakanj in Bosnia and Herzegovina. So, the increased number of alleles can be explained as a consequence from the use of mixed initial seed material, collected at population level for the establishment of plantations which preceded the establishment of the seed orchard.

The total genetic differentiation among phenogroups was considerably lower than the values defined for natural populations in the previous research (F_{ST} = 0.050) and it can be characterised as small. The value of F_{ST} reflects the differences in the allele frequencies among phenogroups, which is mostly low. The explanation of the small genetic differentiation could be the long-term crossing of parental individuals of all phenogroups within natural populations and the plantations in which seed material was collected for the establishment of the Godovik seed orchard. The seed material was collected at the plantation level, without previously controlled hybridisation. So, the pollinator in the free hybridisation process could be any "functionally male" individual in the plantation, regardless of the phenogroup. Taking into account the high degree of free crossing in forest trees (3, 19), it can be concluded that gene flow among phenogroups by wind dispersed pollen continued for a long time period in natural populations and plantations, causing the decrease of the degree of divergence among phenogroups, which can be detected by the analysis of isoenzyme systems.

The frequency of the allele ***Dia-C1*** is interesting, i.e. in the majority of phenogroups it is below 10%, while in phenogroup "F", it reaches as much as 50%. Also, the frequency of the allele ***Dia-C2*** exhibits exceptionally high value (**Table 2**), which is rather interesting, because in all previous analyses of Serbian spruce genetic structure, this gene locus was monomorphic, i.e. the allele ***Dia-C2*** was not detected.

In addition to the decrease of degree of differentiation among phenogroups, their intrapopulation crossing also caused the increase of total variability, so the orchard progeny had a greater number of polymorphic genes and heterozygous individuals and the degree of inbreeding was exceptionally low (22, 23). It differs from the results of earlier studies of the trees outside the orchard, where Serbian spruce showed a high inclination to autogamy (25). This could also possibly explain the new allele on the locus ***Dia-C***, and most probably this allele was the consequence of the gene flow that also happened outside the plantation, i.e. could be a result from an interspecific hybridisation, which is not uncommon for this species (20, 27).

In the majority of phenogroups (except for phenogroup "A") the estimated heterozygosity was higher than expected (**Table 3**), which indicated negative value of the fixation index of the analysed polymorphic loci, higher percentage of heterozygotes and an intensive selection against inbreeding individuals. These data are in agreement with the previous conclusions on the very intensive inbreeding depression of Serbian spruce (8, 15). The positive values of the fixation index in phenogroup "A", which are the consequence of the lower estimated heterozygosity on the loci ***Gdh-A*** and ***Mdh-B***, prompts for potential presence of inbreeding within this phenogroup. This could possibly be explained by a degree of crossing incompatibility of these individuals with the members of other phenogroups. The value of genetic distance (*D*) also refers to a similar conclusion.

Genetic distance (*D*) (17), between Serbian spruce phenogroups ranges between 0.005 and 0.022, in average 0.011, which can be characterised as intraspecific variability (**Table 4**). The values of *D* among all phenogroups do not exceed 0.2, i.e. they hardly attain 0.02, which is the critical value of this parameter for subspecies and varieties (18).

TABLE 4

Genetic distance

Phenogroupe	A	B	C	D	F
A	xxxx				
B	0.022	xxxx			
C	0.007	0.016	xxxx		
D	0.014	0.005	0.015	xxxx	
F	0.012	0.006	0.008	0.006	xxxx

The smallest genetic distance was registered between phenogroups "B" and "D" (0.005), while the distance of

phenogroup "F" from them was slightly greater (0.006). Phenogroup "C" was somewhat more distant from the first homogeneous group (0.016 and 0.008), while the distance of phenogroup "A" from the other phenogroups was considerably greater.

Conclusions

The assessment of Serbian spruce genetic diversity at molecular level within an *ex situ* seed orchard can be considered as a pioneer undertaking. In addition to the defining of the degree of genetic variability within the orchard, the results of such analyses also offer an insight, although limited, into Serbian spruce diversity in natural populations that can be considered as parental for the trees in the orchard. Consequently, the existence of such a seed source makes the collection of samples easier. In addition, gene pools of populations from different sites are positioned at one only location on an accessible terrain. The phenotypically determined varieties and types, separated in blocks and sited in more or less the same ecological conditions in the Godovik seed orchard, provides a very good research base for defining the Serbian spruce intraspecific genetic diversity.

Molecular analyses, in addition to a conservation objective, i.e. the study of the degree of intraspecific variability, also have a breeding objective, namely to define the degree of genetic control of desired phenotype traits and their unification by the use of an optimal model of controlled hybridisation. Taking into account that the variability at molecular level does not have to be related to the variability in the biological functioning and phenotype characters (26) as well as the fact that the value of genetic distance at molecular level is a very good indicator of the success of future hybridisation, the controlled hybridisation model based on the results of the analysis of genetic diversity of phenogroups can offer an optimal starting point for further breeding process for the concerned species.

The establishment of an adequate hybridisation model including the above mentioned phenogroups, in order to unify their positive ornamental, productive and ecological characteristics would be the next phase of enhancement of the species' genetic structure and utilisation. As the success of controlled hybridisation of different varieties depends also on their genetic compatibility, the data on the phenogroup genetic distance are highly useful. The controlled hybridisation in the Godovik orchard, which would result in the development of inter-group hybrids, could be of high significance for forestry and horticulture. Based on the genetic distance values, it is recommended to cross the genotypes of phenogroups "B" and "D" as the genetically most similar ones, This would possibly result in a new variety to be characterised by double-topped semi-dwarf trees. Along with this model of hybridisation all other hybridisations among phenogroups "B","D", "F" and "C" will likely be successful, with the following resultant phenotypes being expected: double-topped semi-dwarf trees ("B" x "D"), semi-dwarf trees with silver crown ("F" x "D"), semi-dwarf trees with narrow-pyramidal crown ("D" x "C") and double-topped trees with silver crown ("B" x "F").

The crossing of phenogroup "A" with other phenogroups in the seed orchard is not recommended, because the great genetic distance indicates a low degree of hybridisation success. If sufficient financial means are available, this type of hybridisation can also be carried out in order to verify the above stated hypothesis.

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