

ALLELE POLYMORPHISM OF *NAD1* GENE OF THE SERBIAN SPRUCE MITOCHONDRIAL GENOME

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Serbian spruce (*Picea omorika* /Panč./Purkyne), as the Balkan Peninsula endemic and the Tertiary relic, is a species whose survival is threatened by the constant restriction of its range caused by the global changes of environmental conditions and the adverse human impacts. The Serbian spruce seedling seed orchard at Godovik represents the base for the improvement of the production of the selected seeds of this species, which can be used as the initial material for the extension of its range. The allele polymorphism of the mitochondrial *nad1* gene was analysed in five different Serbian spruce phenogroups of which the orchard is established. The obtained results are a contribution to a closer study of the causes of the postglacial intraspecific differentiation of Serbian spruce and the creation of the above phenogroups. The study results are significant for further breeding of this species based on the better knowledge of the

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genetic structure of the species, its directed utilisation and the widening of its range.

Key words: Serbian spruce, mtDNA, intraspecific differentiation

INTRODUCTION

The efficiency of different measures of germplasm conservation of this valuable species depends primarily on the degree of information on its genetic variation. During the last fifteen years, the demand for codominant, locus specific and multiallele DNA markers has been considerably increased, because they can be applied to the population genetics research, mapping and marker assisted selection, in the aim of defining the association of genes and phenotypic traits.

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, KEATHLEY (1996). By the parallel analysis of isoenzyme markers and quantitative traits, KUITTINEN *et al.* (1991) studied the genetic structure of a Serbian spruce natural population at Stolac, near Višegrad in Bosnia and Herzegovina, and of the artificially established plantation in the Arboretum Punkaharju in Finland. The results of the above studies pointed to the extremely high level of genetic variation, in both populations. BALLIAN *et al.* (2005) analysed the genetic variation of five natural Serbian spruce populations from Bosnia and Herzegovina, by the analysis of 12 enzyme systems and 16 gene-loci. The results of these studies point to the significant differences in the genetic structure among the analysed populations from the area of Višegrad. It was concluded that the seeds and cones should be declared separately before sending to the market, which had not been practiced, as the declaration referred only to the Višegrad provenance. BALLIAN *et al.* (2006) analysed the genetic variation of 12 natural populations and one plantation from Bosnia and Herzegovina, by the analysis of 16 isoenzyme loci. Most of the populations had a significant number of heterozygotes, which pointed to a potent selection against inbreeding individuals.

The mitochondrial *DNA* is the most often used molecular marker in phylogeographic researches, thanks to its wide range of intraspecific polymorphism. It was successfully applied to the phylogeographic and population researches of animals and the human population (AVISE 1994; SELSTAD *et al.* 1998; TABERLET *et al.* 1998; HEWITT 1999), while its use in plant populations is still not common. Mitochondrial *DNA* is exclusively inherited maternally, which means that different variants of *mtDNA* are dispersed by seed, and not by the pollen. The research of mitochondrial gene polymorphism is a good base for the study of the process of migration and geographic differentiation in a species population (SPERISEN *et al.* 2001). *MtDNA*, has more disadvantages as a marker of plant genome than as a marker of animal genome, due to the poorer substitution of nucleotides, i.e. the lower intraspecific variability, heteroplasmy, wider range of recombination among the molecules and a considerably greater *mt* genome.

However, the application of mtDNA markers is justified in conifers, because chloroplast DNA is inherited paternally. Numerous researches, by the application of mitochondrial DNA, define the relevant factors which determine the actual distribution intra- and inter- species, such as: (a) location of glacial refuges in Italy, on the Arabic and Balkan Peninsulas and the effect of migration processes and corridors; (b) the role of historical factors (especially the mechanisms of seed and pollen dispersion); and (c) human impact on different species. The common history of different forest tree species migration and their phylogeographic distribution was also defined (PETIT *et al.* 2003).

MATERIAL AND METHODS

The research was performed on the material collected in the Serbian spruce seedling seed orchard in the village Godovik, Municipality Požega (TUČOVIĆ & ISAJEV 1986). In the experiments with the 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 was exposed to identical environmental conditions and treatments (HATTEMER & ZIEHE 1997). The planting pattern in the seedling seed orchard at Godovik satisfies the above criteria, which shows that the genetic research within this unit of *ex situ* conservation is justified.

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

The intensive research of the plantations started by the classification of trees into phenogroups which were considered to be significant for forestry and horticulture (ISAJEV 1987):

- **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 revealing, selecting and defining of different phenogroups within the three above plantations, which preceded the orchard at Godovik, was a significant

step in the study of Pančić's Serbian spruce intraspecific variation, which had previously been characterised as exceptionally uniform. The phenotypic determination of different intra-specific varieties and types showed the opposite, and it opened numerous questions on the degree of Serbian spruce genetic diversity, genetic control of the phenotypic expression, the degree of inheritance of characteristic traits and the new revealed potential for further breeding of the species (ISAJEV 1991, ISAJEV *et al.* 1992).

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

The Godovik 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. Phenogroup "E" is by definition characterised by dwarf individuals, height up to 0.7 m, which is not the case at Godovik orchard at the age of twenty, where the trees reach the height of up to even several metres. The individuals in phenogroup "G" are not covered by this research, because the location of their blocks during the sampling period (January 2006) was extremely difficult to access.

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, from which the samples could be collected.

The analyses were carried out on the sample of 15 trees from five half-sib lines within five phenogroups (1A3, 1B1, 1C2, 1D1 and 1F7). In January 2006, fresh branchlets with dormant buds were collected and transported to the laboratories of the Department of Phytology, Faculty of Forestry, Technical University of Zvolen, Slovak Republic.

The total genome DNA was isolated from 10–20g (approximately three Serbian spruce needles) of oven dry plant tissue. DNA isolation was based on the modified protocol for rapid isolation of small DNA quantities from the fresh leaf tissue (DOYLE & DOYLE 1987).

The internal fragment of the second intron of the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene was amplified by using Polymerase Chain Reaction. PCR followed the proposal reported by SPERISEN, *et al.* (2001), applied to Norway spruce, with some modifications of the mixture composition (Table 1). PCR amplification was performed by PTC-100 thermal cycler (MJ Research), total volume 20 µl, with primers *nad1F* (5'3'; CTC TCC CTC ACC CAT ATG ATG) and *nad1R* (5'3'; ACA AAG CCC CTT TGA GGG). The profile of the process was the following: 3 minutes denaturation at 94°C and 26 cycles of denaturation of 1 minute at 94°C, 1 minute annealing at 57°C, 2 minutes extension at 72°C.

Table 1: Mixture content used for PCR

Component	Starting solution	Final concentration	Quantity in reaction (μ l)
Buffer (with Mg Cl ₂)	10x	1x	2.5
dNTP mix	5 mM	100 μ M	0.5
Primer nad1F + nad1R	10 μ M	200 nM	0.5
Taq polymerase (Sigma)	5 U/ μ l	0.04 U/ μ l	0.1
H ₂ O	-		15.4
DNA 1:5			1
Total reaction volume (μ l)			20

The PCR products were digested with the blunt-end restriction enzyme *EcoRV* (*E059 Restriction Endonuclease*) to increase resolution of sizing (SPERISEN, *et al.* 2001). Electrophoretic separation was performed on 2% agarose gel. The visualisation of fragments was performed by the method of ethidium bromide.

Statistical data processing was carried out by the programme *POPGENE* Version 1.31 (YEH, YANG & BOYLE 1999). Nei's measures of genetic distance (NEI 1978) has been applied.

RESULTS AND DISCUSSION

In the analysed plant material, six different alleles were detected on *nad1* gene-locus (1, 2, 3, 4, 5, 6).

The rare fragments 5 and 6, which migrate through the gel with the highest speed, are present in the individuals of the phenogroups "B", "D" and "F". Other alleles, whose migration is slower (1, 2, 3, 4), are present in the genotypes of all the study phenogroups. According to METTLER and GREGG (1969), a great number of alleles on a definite gene-locus can be without a current function or even harmful to the population from the aspect of adaptation to the current environmental conditions, but they are retained in order to enable the evolution and further adaptation, which can be one of the explanations for the occurrence of the maternally inheritable, rare alleles. They form the base of the further process of evolution and only the most successful alleles can be incorporated in the population and included in the evolution trends (TIGERSTEDT & YAO 1997). So, the rare alleles have an essential role in future processes of the population adaptation, but they are, as it is supposed, of low significance to the formation of the today's adapted natural and anthropogenically improved population. Generally, the rare alleles have a low effect on the genic diversity.

Genic diversity, after NEI (1973), shows a considerable variation across the study phenogroups (*Table 2*). The highest value of this parameter, which indicates the allele diversity in the gene pool of the analysed locus within the phenogroup, is found in phenogroup "C" (0.7022), in which this value nears the maximum of 1, which means such a genetic structure of the population in which each randomly selected gene represents a unique allele. The values of genic diversity of Serbian spruce from the Godovik seedling seed orchard cannot be compared to the values of natural populations, because the polymorphism of *nad1* gene in Serbian spruce has not been researched to date.

Table 2: Genetic Variation Statistics for Nad1 locus

Phenogroup	Sample Size	Nei's (1973) gene diversity
A	15	0.3292
B	15	0.1244
C	15	0.7022
D	15	0.5600
F	15	0.3867

The analyses of mitochondrial genes are most often the base for the study of the migration process and geographic differentiation of the species populations (SPERISEN *et al.* 2001), so the *nad1* gene of Serbian spruce mitochondrial genome was analysed in the aim of studying the differentiation course of the species populations during the post-glacial period and the creation of the phenogroups.

The determined diversity on the study locus points to a significant degree of intraspecific variation of the species which is today characterised by small, isolated and restricted populations, therefore it can be considered as the consequence of the effect of the Serbian spruce former widespread natural populations. As phenogroup "C" is characterised by the highest values of genic diversity, and as the individuals classified in this phenogroup have the typical Serbian spruce habit, it can be hypothesised that it represents the conserved part of the gene pool of the Serbian spruce former widespread populations.

The genetic distance of the study phenogroups was estimated based on the values of genetic distance after NEI (1978). The genetically most similar phenogroups are "B" and "D". The minimal deviation from the first homogenous group is shown by phenogroup "F", somewhat more by phenogroup "C", whereas

the distance between phenogroup “A” and other phenogroups is the greatest (*Table 3, Figure 1*).

The genetic distance is the parameter which can offer considerable information from the aspect of taxonomy, phylogenesis and phylogeography. NEI (1976) measured the values of genetic distance characterising the species, subspecies and varieties. According to his research, the species are distinguished by the genetic distance from 0.1 to 1.0, while the subspecies and varieties are characterised by the values from 0.02 to 0.2. After NEI (1978), the genetic distance (*D*), between the study Serbian spruce phenogroups is between 0.0379 and 1.2173, with the average of 0.5460, which can be characterised as the intraspecific variation. The genetic distance among all phenogroups, except phenogroup “A”, ranges between 0.02 and 0.2, i.e. they belong to intraspecific variation. The genetic distance between phenogroup “A” and the other phenogroups, except the phenogroup “C”, has the values which exceed the maximum 1.0 characterising the species.

Table 3: Genetic distance according to Nei (1978)

Phenogroups	A	B	C	D	F
A	xxxx				
B	1.2173	xxxx			
C	0.7073	0.1741	xxxx		
D	1.5796	0.0379	0.2013	xxxx	
F	1.2146	0.0421	0.1905	0.0952	xxxx

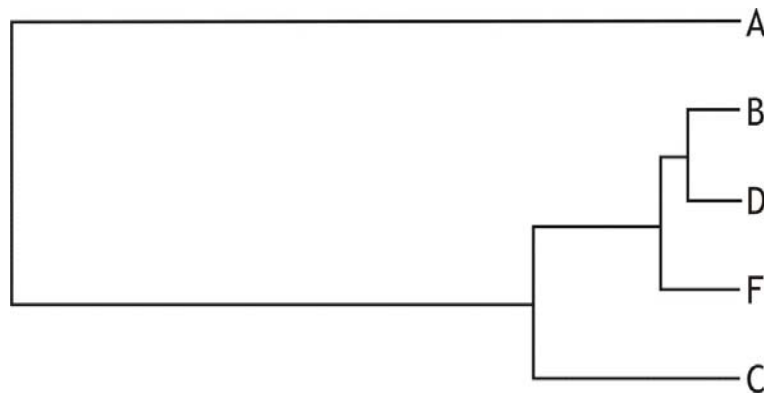


Figure 1: Dendrogram of Genetic Distance according to Nei (1978)

Phenogroup "A", defined as the variety "*borealis*" whose individuals are very similar to Norway spruce, is at the maximal genetic distance from the other study phenogroups, which indicates its greater genetic similarity with Norway spruce, compared to other phenogroups. Also, the values of genetic distance exceeding the limit of intraspecific variation, after NEI (1976), point to the probable genetic similarity of this phenogroup with Norway spruce. Taking into account the high capacity of Serbian spruce for interspecific hybridisation with the species in *Picea* genus (ROULUND 1971; VIDAKOVIĆ 1963), and the fact that Serbian spruce most often occurs in the mixed stands with Norway spruce, it can be inferred that the individuals in phenogroup "A" represent interspecific hybrids of spruce and Serbian spruce. This hypothesis should be researched by further study of maternal genome variation of both species, but the molecular analyses should also be performed, which would elucidate the interspecific variation, as well as the characteristics of the paternal genome (chloroplast *DNA*).

The genetic distance of the marker gene-locus between the groups or individuals characterised by different phenotypes is an indirect method for the assessment of genetic control of phenotypic expression. A significant genetic distance between phenogroups is a sufficient indicator of the existence of the genetic control of the expression of the concrete trait, which, of course, does not mean that the study gene-locus is responsible for the concrete phenotypic trait (HATTEMER & ZIEHE 1997). Based on the above and based on the values of the genetic distance between phenogroups determined in this study, it can be concluded that the characteristic traits which define the Serbian spruce phenogroups are genetically determined, but it is not possible to determine to which degree. The explanation of the phenogroup differentiation could be found in the ecological-genetic phenomena, such as the "carryover" effect (JABLONKA *et al.* 1995), because it is very likely that the above mechanism is under some degree of genetic control.

To be able to survive, a population has to adapt to the current environmental conditions, which means that most of the individuals in the population have to take up the phenotype which enables the survival in the current conditions (METTLER & GREGG 1969). The changes of environmental conditions bring about the phenotype changes of the population members. In addition to the changes of genetic structure by recombination and mutation, forest trees also have the "carryover" mechanism which causes the creation of the new, adapted phenotypes. The "carryover effect" is an adaptive mechanism in the evolution processes, which fills the gap between the short-term individual adaptation and long-term evolutionary adaptation. This phenomenon implies the presence of the created phenotypes through several generations despite the change of the environmental conditions which caused its creation. This phenomenon can be considered as the concept of plasticity with the added time dimension, because thanks to this phenomenon, the new phenotypes remain stable throughout several generations, clonal and seedling.

Based on the above, the Serbian spruce intraspecific differentiation can be compared to the process of allopatric speciation (CHIRAS 1991). Due to the geographic isolation and sharp changes of environmental conditions, the former widespread Serbian spruce populations started to divide. The small populations fragmented gradually, colonised the most favourable sites and thus became separated by ecologically unfavourable areas. The period of isolation was followed by the fragmentation into still smaller populations, or the so-called demes (GILMOUR & GREGOR 1939). Each deme retained the same genic composition as the ancestral population. The ecological barriers of gene exchange and the different conditions of development were the base of the divergence between them. Each isolated deme was modified by the effect of different micro-ecological conditions, and the modifications persisted during several generations. The demes started to differ, although they still remained open to the exchange of genes. The above scenario can be the model for the explanation of the formation of Serbian spruce phenogroups.

The phenotypic traits essential for the designation of the above varieties and types, to some extent, point to the ecological conditions to which the small, isolated populations were exposed. The individuals of the phenogroup "C" remained on the typical Serbian spruce sites, steep rocks and deep gorges with deep snow cover, and kept the characteristics of the typical habit, grand height, narrow, pyramidal crown and short branches. Phenogroup "A" consists of the trees with wide crowns and with branching similar to Norway spruce, which points to their existence in the mixed stands with Norway spruce where, by widened crowns, they struggled against the competition of the admixed species. The dichotomy of trees in phenogroup "B" can be related to the former intensive influence of some biotic or abiotic factors which caused the death of the apical bud. The trees developed the defence mechanism and kept it after the disappearance of the causal agent. Variety "D" is characterised by semi-dwarf trees, height up to 1.8 m, which is probably the consequence of extremely poor site conditions (deep shade, high level of nitrogen in the soil), in which the height increment was significantly reduced. The silvery crown appearance of the individuals in phenogroup "F", which is the consequence of the needles pointed upwards on current-year and second-year branchlets, is most likely the response to the high air humidity, high precipitation sum and deep snow cover. The greatest number of stomata, which enable the transpiration and ventilation, occurs in the lower epidermis, which caused the modification of the direction of needle growth.

CONCLUSION

Serbian spruce phenogroups, incorporated in the Godovik seedling seed orchard, can be considered as the indicators of intraspecific variation. Each phenogroup is characterised by a specific habit, which can be considered hereditary, as it is present already in two known generations in different ecological

conditions. Consequently, the hypothesis on the genetic determination of the specific phenogroup traits is fully justified.

Based on the results of the analysis of allele polymorphism of *nad1* gene of the mitochondrial genome, it can be concluded that there is a degree of genetic control of phenotype expression in different Serbian spruce phenogroups. This form of intraspecific variation could be explained by the adaptive-ecological phenomena, whose genetic determination has not been completely explained yet, but it exists. Based on the genetic distance from the other phenogroups, the phenogroup "A" can be supposed to represent the inter-species hybrid of Serbian spruce and Norway spruce, which opens a new topic for the future research of Serbian spruce intraspecific variation.

The study results presented in this paper are significant for the closer investigation of the genetic structure of this endemic and relic species. The study of the Serbian spruce genetic map is a contribution to the research directed to the elucidation of the nature of the causes of Serbian spruce genetic specificity, as well as to the selection of the suitable methods for the conservation of its diversity during the process of global environmental changes.

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**ALEJNI POLIMORFIZAM *NAD1* GENA MITOHONDRIJALNOG
GENOMA OMORIKE**

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Izvod

Omorika (*Picea omorika* /Panč./Purkyine), kao endemit Balkanskog poluostrva i tercijarni relik, predstavlja vrstu čiji je opstanak ugrožen konstantnim sužavanjem areala usled globalnih promena uslova sredine i negativnih posledica uticaja čoveka. Generativna semenske plantaža omorike u Godoviku predstavljaju osnovu za unapređenje proizvodnje selekcionisanog semena ove vrste, koje se može upotrebiti kao polazni materijal za proširenje njenog areala. Pet fenogrupa omorike: „A“ - varijetet „*borealis*“, B“ - varijetet „*semidihotomy*“, „C“ - varijetet „*serbica*“, „D“ - varijetet „*nana*“, „F“ - tip „*argentea*“, ugrađene u generativnu semensku plantažu u Godoviku, su pokazatelj unutarvrzne varijabilnosti ove vrste. Svaka fenogrupa karakteriše se specifičnim habitusom, koji je verovatno pod genetikom kontrolom s obzirom na to da je prisutan već u dve poznate generacije i to različitim ekološkim uslovima.

Na osnovu rezultata analize alelnog polimorfizma *nad1* gena mitohondrijalnog genoma može se zaključiti da postoji određen stepen genetičke kontrole fenotipske ekspresije kod različitih fenogrupa omorike. Objašnjenje nastanka ovog oblika unutarvrzne varijabilnosti treba potražiti u adaptivno-ekološkim fenomenima, čija genetička determinacija još uvek nije u potpunosti objašnjena, ali je izvesno da postoji. Na osnovu genetičke udaljenosti od ostalih fenogrupa, za fenogrupu «A» može se pretpostaviti, da predstavlja međuvrtni hibrid omorike i smrče, čime se otvara novo pitanje za buduća istraživanja unutarvrzne varijabilnosti omorike.

Dobijeni rezultati daju doprinos bližem upoznavanju uzroka nastale postglacijalne unutarvrzne diferencijacije omorike i nastanak ovih fenogrupa. Dobijeni rezultati su od značaja za dalje procese oplemenjivanja ove vrste na osnovama boljeg poznavanja njene genetičke strukture, njeno usmereno korišćenje i proširenje areala.

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