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Review of investigation of variability of nad1 gene intron B/C of mitochondrial genome in Scots pine (*Pinus sylvestris L.*)

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Abstract

Background and Purpose: *Scots pine (*Pinus sylvestris *L.) is the most extensively distributed pine, covering the whole Eurasian continent. The detection of genetic variability of Scots pine is of greate importance from the evolutionary point of view as well as for genetic conservation. Mitochondrial genome in Scots pine is maternally inherited and dispersed through seeds. Molecular studies have indicated high genetic diversity in Scots pine genome, allowing the detection of variation between and within population. Mitochondrial genome possesses variable regions in the gene encoded NADH dehydrogenase. The variable region is nad1 B/C intron. The different haplotypes of nad1 B/C intron allowed confirming the Iberian refugia in Holoecen and confirming the genetic diversity between different stands of Scots pine in Poland. The purpose of this study was to find out the genetic variability of nad1 B/C intron in two International Scots pine provenance experiments and native Scots pine provenance in Croatia in order to detect multiple origin and haplotype variation between provenances.*

Material and Methods: *The samples of Scots pine were taken from two International experiments of provenances, one established in Croatia (22 provenances) and the other established in Hungary (20 provenances) and Croatian native provenance (Forest office Vrhovine, Forest administration Gospić). The samples of needles were collected from randomly chosen trees of each provenance. Eight samples per provenance were collected for haplotype analyses. DNA was extracted and amplified with specific primers for nad1 B/C intron of the mitochondrial DNA. PCR products were analyzed using agarose gel electrophoresis and capillary electrophoresis on the bioanalyzer Agilent 2100.*

Results and Conclusion: *We investigated samples from 42 provenances of Scots pine that originated from a broad range in Eurasia and were included in two international provenance experiments, and samples from native Croatian population. A total of 344 individual trees of Scots pine were analyzed. The data were analyzed by agarose gel electrophoresis and by capillary electrophoresis. We did not detect variability in mitochondrial nad1 B/C region in analyzed samples. All analyzed samples were haplotype a. Based on our data, we could conclude that none of the tested samples originated from the Iberian glacial refugia. All tested samples could have the origin in glacial refugia placed in Mediterranean, Balkan region or in the northeastern Europe. More variable region of the nuclear DNA and the mitochondrial DNA should be tested on a greater number of samples to obtain more informative data.*

TABLE 1

Locations of analyzed Provenances of Scots pine.

no	Country	Settlement / Forest disctrict	Latitude (N)	Longitude (E)	Number of sample
International experiment of provenances, Hungary					
1	Russian Federation	Horinszk	52° 07´	109° 46´	8
2	Russian Federation	Ayan	55° 31´	138° 04´	8
3	Kazakhstan	Beszkaracsajszk	47° 00´	82° 00´	8
4	Russian Federation	Liszin	69° 15´	31° 15´	8
5	Russian Federation	Pecsenga	60° 25´	31° 15´	8
6	Ukraine	Dubrovitsa	51° 40´	26° 40´	8
7	Russian Federation	Orehovo-Zujevo	55° 47´	38° 25´	8
8	Russian Federation	Vesenszkaja	49° 40´	41° 45´	8
9	Russian Federation	Glazov	58° 08´	52° 46´	8
10	Russian Federation	Duvan	55° 19´	57° 00´	8
11	Russian Federation	Szuzun	53° 55´	82° 04´	8
12	Russian Federation	Boguchany	58° 23´	97° 26´	8
13	Estonia	Elwa	58° 16´	26° 25´	8
14	Slovak Republic	Malacky	48° 30´	17° 04´	8
15	Czech Republic	Plzen	49° 49´	13° 19´	8
16	Germany	Eberswalde	52° 50´	13° 50´	8
17	Hungary	Pornóapáti	47° 20´	16° 28´	8
18	Poland	Ruciane	53° 51´	21° 31´	8
19	Turkey	Catacik	40° 00´	31° 10´	8
20	Bosnia and Herzegovina	unknown	44° 07´	18° 36´	8
International experiment of provenances, Croatia					
21	Russian Federation	Roshchinskaya Dacha	60° 15´	29° 54´	8
22	Russian Federation	Kondezhkoe	59° 58´	33° 30´	8
23	Russian Federation	Serebryanskoe	58° 50´	29° 07´	8
24	Latvia	Silene	55° 45´	26° 40´	8
25	Poland	Milomlyn	53° 34´	20° 00´	8
26	Poland	Suprasl	53° 12´	23° 22´	8
27	Poland	Spala	51° 37´	20° 12´	8
28	Poland	Rychtal	51° 08´	17° 55´	8
29	Poland	Bolewice	52° 24´	16° 03´	8
30	Germany	Neuhaus	53° 02´	13° 54´	8
31	Germany	Betzhorn	52° 30´	10° 30´	8
32	Germany	Lampertheim	50° 00´	10° 00´	8
33	Belgium	Ardennes	50° 46´	4° 26´	8
34	France	Haguenau	48° 49´	7° 47´	8
35	Sweden	Sumpberget	60° 11´	15° 52´	8
36	Czech Republic	Zahorie	48° 46´	17° 03´	8
37	Hungary	Pornóapáti	47° 20´	16° 28´	8
38	Montenegro	Maočnica	43° 10´	19° 30´	8
39	Bosnia and Herzegovina	Prusačka Rijeka	44° 06´	17° 21´	8
40	Turkey	Catacik	40° 00´	31° 10´	8
41	Bosnia and Herzegovina	Zavidovići	44° 21´	18° 15´	8
42	Serbia	Bosiljgrad	42° 29´	22° 29´	8
Native provenance Vrhovine, Croatia					
43	Croatia	Vrhovine	44° 44´	15° 45´	8

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) has a very great area of distribution. According to Ruby and Wright *(1)* it occupies almost the whole northern part of Euroasia. The distribution range of Scots pine covers approximately 135 degrees of geographic longitude and approximately 30 degrees of latitude. The northern border of Scots pine occurs up to 70° 20' northern latitude. The southern border of its range is placed at 38° 34' northern latitude *(2)*. It grows naturally from Scotland and Scandinavia to the Pyrenees, in Southern and Central Europe, and in northern Greece. In Asia, it extends to central Turkey, to the Caucasus, northern Manchuria and the Okhotsk Sea *(3)*. In Croatia, Scots pine grows in isolated areas. It is found in mountain region (Lika) and near the river Drava (Prekomurje) *(4)*. Artificially, it was distributed in forest cultures and, in the relation with other conifers, it was represented by 22% in coniferous forest cultures in Croatia *(5)*.

The largest migration of Scots pine in Europe occurred during the Pleistocene *(6)*. Climatic fluctuations of glacial and interglacial periods at the Pleistocene had a great impact on morphological and ecological differentiation and preservation of distinctive genotypes of Scots pine *(7, 8)*. During the last glacial period, Scots pine survived in Europe in scattered and restricted refugial areas. Data on macrofossil remains, palynological analysis, vegetating modeling and DNA survey have shown that the glacial refugia were present in the Mediterranean area, around the Alps, the Hungarian plain and the Danube region *(9)*. Genetically distinct glacial refugia were reported in Asia Minor, as well as in the northeastern Europe and the Baltic region *(10)*.

DNA-based marker systems have become of common use for evolutionary and population studies of Scots pine *(11)*. The DNA marker placed in mitochondrial DNA is of particular interest because of its unique mitochondrial characteristic as a plant organelle. The mitochondrial DNA behaves as a single haploid gene, which accounts for the absence of meiotic recombination *(12)*. In conifer, there is maternal inheriting and dispersion between populations only by seed flow *(13, 14, 15)*.

Variable region in the mitochondrial genome is *nad*1 B/C intron of NADH dehydrogenase. Genetic variability in the *nad*1 B/C intron clearly distinguished the Scots pine of Iberian Peninsula refugia from those of the rest of Euroasia *(9, 16)*. The different haplotypes of *nad*1 B/C intron are characteristic for Italian refugia and the refugia located around the Alps (16). In Polish provenances of Scots pine, four haplotypes of *nad*1 B/C intron were determined *(17)*.

The purpose of the present study was to analyze the variability in *nad*1 B/C intron from the mitochondrial region of individual trees from two international Scots pine provenance experiments, i.e., from Croatia and Hungary, and in native Croatian population. The result of the investigation should give more data about complex refugial origin site and haplotype variation between geographically widespread provenances of Scots pine.

MATERIAL AND METHODS

Plant material

Samples were collected in two international provenance experiments of Scots pine, one located in Croatia and another in Hungary (Figure 1, Table 2). Provenance variations of Scots pine were investigated in a national and international experiment upon recommendation of the International Union of Forest Research Organizations (IUFRO) *(18, 19)*. In Croatia, the international provenance experiment of Scots pine was established in

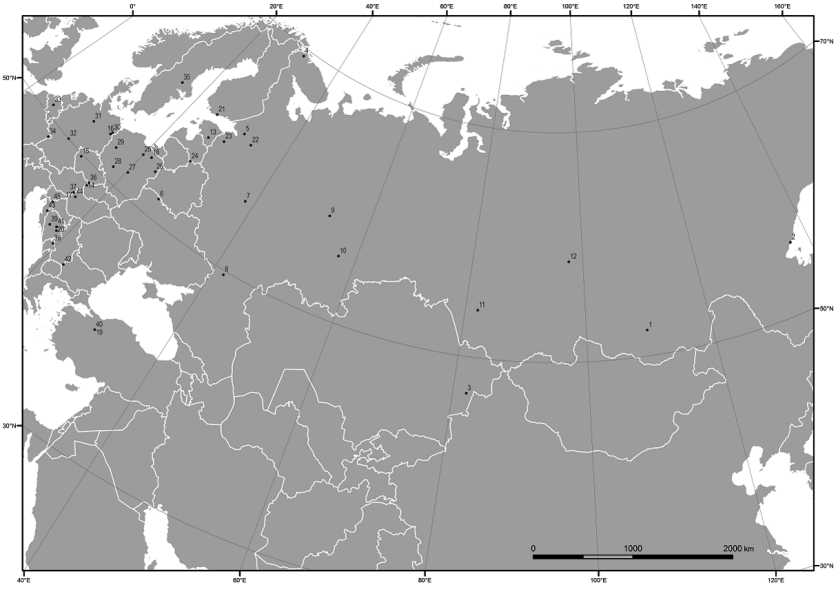

Figure 1. *Geographical distribution of analyzed provenances of Scots pine and location of planting sites. Details of provenances and planting sites are given in Table 1 and Table 2, respectively (see respective number).*

TABLE 2

Data about planting sites of the IUFRO provenance experiments of Scots pine.

No.	Country	Locality	Latitude (N)	Longitude (E)	Seed collection (year)	Sowing (year)	Planting (year)
44	Hungary	Egyházashetye	47° 10´	17° 07´	1975	1976	1978
45	Croatia	Drenovac	45° 33´	15° 22´	1978–1980	1982	1985

1982 on locality Drenovac, Forest office Duga Resa, Forest administration Karlovac *(4, 20)*. In this experiment, 22 provenances from 13 European countries (Russian Federation, Latvia, Sweden, Poland, Germany, Belgium, France, Czech Republic, Hungary, Turkey, Bosnia and Herzegovina, Montenegro and Serbia) were included, and it was supported and managed by Forest Research Institute. In Hungary, the international provenance experiment Jastrebarsko (present name Croatian Forest Research Institute) of Scots pine was established in 1978 in Egyházashetye, Forest District Sárvár. The seeds originated from 85 provenances from 11 countries (Russian Federation, Estonia, Kazakhstan, Ukraine, Poland, Germany, Slovak Republic, Czech Republic, Hungary, Turkey and Bosnia and Herzegovina). For this study, 20 provenances were chosen for sample collections. The natural population of Scots pine placed in Vrhovine, Croatia, was included in the study.

The samples of 43 provenances were spread from the northern places in Euroasia, beyond the Arctic circle (approximately at 69° northern longitude) to southern sites in Euroasia (approximately at 39° northern longitude). The northernmost provenance was Roshchinskaya Dacha (60° 15´N, 29° 54´E) in Russia and the southernmost provenance was Catacik (40° 00´N, 31° 10´E) in Turkey. The westernmost provenance was Haguenau (48° 49´N, 7° 47´E) in France and the easternmost provenance was Ayan (55° 31´N, 138° 04´E) in Russia. Table 1 summarizes names, geographical coordinates and the number of samples of each provenance used in the study. Figure 1 shows the geographical distribution of provenances in Eurasia. The needles were sampled from 8 randomly selected individuals from each of 43 provenances.

DNA extraction

DNA was extracted from fresh needles (184 Croatian samples) and frozen needles (160 Hungarian samples) according to the protocol of Doyle and Doyle *(21)*. The quality and quantity of isolated DNA was checked on 0.8% agarose gel (Sigma, USA) using the DNA Ladder Lambda DNA/*Hin*dIII Fragments (Invitrogen, USA).

DNA amplification

Amplification was carried out according to Soranzo et al. (16) with modifications. The volume of 25 μL reactions containing 1x PCR buffer (Invitrogen, Germany), 200 mM each dNTPs (Invitrogen, Germany), 8 pmol each primer (nad1H and nad1I, Invitrogen, Germany) and 1.5 units of Taq DNA polymerase (Invitrogen, Germany). Thermocycling was performed in a PTC 100 Thermocycler (MJ Research, USA) in 0.20 ml tubes (Eppendorf, Germany). The thermal profile was the following: 4 min of an initial denaturation step which was followed by 30 cycles of 94 °C (30 sec), 56 °C (30 sec) and 72 °C (30 sec) and a final extension step at 72 °C (5 min).

DNA analyses

To confirm the amplification of a fragment, PCR products were analyzed by electrophoresis on 2.5% agaose gel (Sigma, USA) in 1X TBE buffer (Gibco, USA) stained with SYBER Safe DNA gel stain (Invitrogen, USA) according to manufacturer's instructions. The molecular weight of amplified bands was estimated using O´RangeRuler 50 bp DNA Ladder (MBI, Fermentas, Lithuania). The length of fragments was detected by capillary electrophoresis on bioanalyzer Agilent 2100 (Agilent Technologies, USA) using Agilent DNA 1000 kit (Agilent Technologies, USA).

RESULTS AND DISCUSSION

In this study we analyzed the variation in *nad*1 mitochondrial gene B/C intron of Scots pine. The samples were taken from two International experiments of provenances of Scots pine, Drenovac, Croatia and Egyházashetye, Hungary. All 22 provenances from Drenovac were analyzed. Twenty provenances from Egyházashetye were chosen for analyses with emphasis on the provenances of the Asiatic part of Euroasia. The Croatian natural provenance of Scots pine from Vrhovine was also included in this research. Neither of those provenances were tested on *nad*1 B/C variation with exception of three provenances (Hagenau from France, Silene from Latvia and Zahorie from Czech Republic *(16)*). Both of the two international experiments of provenances contain samples from Pornoapati (Hungary) and Catacik (Turkey). In analyses, 344 individual trees of Scots pine were investigated. Our data show that the amplified fragments of *nad*1 B/C intron region of the mitochondrial DNA were 218–221 bp long in all analyzed samples. Fragment lengths represent haplotype a in all examined individual trees.

In a study of Soranzo *et al.* the Scots and central European populations contain haplotype a and only one haplotype b was detected in Polish population of Scots pine *(16)*. Differences were shown in Spanish populations where both haplotypes, a and b, were detected *(16)*. The result of research of Italian native population of Scots pine suggests that the Alpine and Apennine populations had for haplotype a *nad*1 gene. Haplotype b was not detected *(22)*. A study of Naydenov *et al.* described detection of haplotype b in Bulgarian population of Scots pine

at low frequency *(10)*. However, the amplification of B/C intron of mitochondrial *nad*1 gene was used to determine genetic diversity level in fourteen different stands of Scots pine in Poland, and four different haplotypes were detected a: 189–222 bp, b: 237–250 bp, c: 223–229 bp and d: 230–236 bp *(17)*. We examined 6 provenances of Poland represented by 48 individual trees and we did not detecte haplotype b, nor haplotype c nor d.

In conclusion, our data suggest that the absence of haplotype b in our analyses excludes the possibility that the origin of tested samples is Iberian Peninsula. The investigated samples, according to the result of determination of only haplotype a, have refugial origin in the Balkan region or in the refugia placed in the northeastern Europe. However, the investigation on more variable loci of the mitochondrial and the nuclear DNA, including more samples, would provide more clearly data about geographical origin and variability between and within provenances of Scots pine from IUFRO International Scots pine provenance experiments as well as the native Croatian provenance.

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