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Genetic Analysis of *Pinus sylvestris* L. and *Pinus sylvestris* forma *turfosa* L. Using RAPD Markers

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Abstract

The purpose of the present study was to determine the level of genetic diversity within and among Ciuc basin, Romania (populations from Mohos and Luci raised bogs in Harghita Mountain and Sumuleu in Ciuc Mountain) *Pinus sylvestris* populations using molecular markers. Two of populations (Mohos and Luci) seem to be the descendants that survived the continental glaciations. Genetic diversity was analyzed by RAPD (Random Amplified Polymorphic DNA). Nine primers were selected for analysis, which generated reproductible bands. On base of presence or absence of homologues bands Nei's gene diversity, the percentage of polymorphic loci and Nei's unbiased genetic distance were calculated. The level of genetic variation among populations was found to be low. For both populations the variation values among populations were higher than within populations. The fossil records and geological historical data explain the extremely low genetic diversity of this species. *Pinus sylvestris* experienced strong bottlenecks during its evolutionary history, which caused the loss of genetic variation. Genetic drift and breeding in post-bottlenecked small populations may be the major forces that contribute to low genetic diversity and genetic differentiation of populations. Human activities may have accelerated the loss of genetic diversity in *Pinus sylvestris.*

Keywords: RAPD, genetic diversity, *Pinus sylvestris*, habitat fragmentation, isolation

Introduction

The species *Pinus sylvestris* probably entered Europe migrating from East Asia, where it predominate from Tercier. Until glacial migrations the southern limit of the area has been fragmented. The isolated populations have been identified as glacial relics; some of this was described as newer species, like *P. hamata* (Stev.) Sosn., *P. armena* Koch, *P. sosnowskyi* Nakai in Caucasus. The classification is based on wood anatomy, secondary chemistry and most recently on molecular phylogenetic studies (DeVerno *et al.*, 1997; Kondo *et al.,* 2000; Xia *et al.,* 2001; Nkongolo *et al.,* 2002; Zhi Yong *et al.,* 2005). Morphological and allometric characteristics have been used to assess genetic variation within this species but these markers are not reliable due to ecological variations (Ilstedt, 1987).

Pinus sylvestris is native to the Ciuc and Harghita Mountains, Romania, and is a specie of ecological importance (Banarescu *et al.*, 1973; Boscaiu *et al.*, 1999). The species was once predominating throughout the Harghita and Ciuc Mountains, too (Banarescu *et al.*, 1973). The population structure undergone dramatical changes due to the glaciations, the introduction of *Picea abies* and the extensive logging. To date only 0.2% of the natural *Pinus sylvestris* stands remain from the pre-settlement stands in these regions. The drastic reduction has resulted in the fragmented distribution of *Pinus sylvestris*. The isolated micropopulations have suffered decrease of genetic diversity, as a result of random genetic drift, reduced gene flow, and reduced habitat quality.

Pinus sylvestris forma *turfosa* is an endangered species, described in the Mohos raised bog in Harghita Mountains. There is only a 20 ha area inhabited by population of this subspecies. Because its rareness and endangered status, it was listed as a protected plant by Harghita County Council. The *turfosa* form is a result of low habitat quality, especially low nitrogen level, increased humidity and moorland.

Fossil and historical data show, that *Pinus sylvestris* population from the Luci raised bog is about 10,000 years old (Tantau, 2006). Buffer zone and neighboring area is a continuous *Picea abies* forest with extensive logging and farming activity, which could seriously affect the *P. sylvestris* population. Both the areas of Mohos and Luci bogs served as refugia during the glaciations. The major part of vegetation from the neighboring areas was eliminated during the glaciations period and repopulated from these refugias starting from the post-glacial periods (Banarescu *et al.*, 1973). Nevertheless, the centuries spent in refugias have marked the populations' genetic reserve and resulted

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in genetic bottlenecks. In addition the two areas are geographically isolated, Mohos occupies a volcano crater in the Ciomad block, and Luci is situated in a depression in Harghita Mountain at 20 km from Mohos. Due to their geographic location and surroundings, this distance makes impossible even a reduced rate of gene flow between populations.

The third population, in Sumuleu Mountain, has smaller habitat size with about 1,5 ha area inhabited with 100-120 individuals, this area showing a higher habitat quality. This population is also native. Between the three populations no gene flow or other connection exists, due to geographic barriers between habitats.

Although the community structure, wood anatomy and phenology of *Pinus sp.* have been extensively studied (Bush, 1991; Deng, 1995), little is known about its genetic background. Conservation genetics has focused on rare and endangered species, however, successful strategies for the maintenance of a species must include understanding the levels and distribution of genetic diversity.

The randomly amplified polymorphic DNA (RAPD) method offers a promising marker system for use in the detection of genetic diversity in population genetics and conservation genetics (Namkoong *et al.*, 1993), having successfully been employed for that purpose in many species. It is expected that the genetic information revealed from this study using RAPD markers will help to provide useful tools for implementation of conservation programs for this endangered species.

Materials and methods

Plant material

Leaf samples were collected from 5-5 plants from three population in Harghita and Ciuc Mountains:a *Pinus sylvestris* population from Luci raised bog, a population from Sumuleu Mountain and a *Pinus sylvestris forma turfosa* population from the Mohos raised bog. Leaves were collected in July 2008.

DNA isolation

The protocol of CTAB total DNA isolation (Doyle and Doyle, 1987) was applied to isolate total genomic DNA from liquid nitrogen frozen leaves. The DNA concentration was adjusted to 20 ng/µl for use in the polymerase chain reaction.

RAPD PCR amplification

Arbitrary primers (12) were used in the RAPD analysis. Primers were synthesized by Bioresearch Technologies, Inc., California. Amplifications were performed using a Corbette thermocycler, programmed for 40 cycles of 94°C for 30 sec, 35°C for 35 sec and 72°C for 1 min 30 sec. An initial denaturating step of 94°C for 45 sec, 41°C for 1 min and 72°C for 2 min and a final extension step of 8 min at 72°C were included in the first four cycles and the last cycles, respectively. Reactions were carried out in a volume of 25 µl, containing 1X PCR buffer, 0,2 mM dNTP, 2 mM $MgCl_2$ 0,5 µM primers, 0,02U/µl Taq polimerase. Amplification products were analyzed by electrophoresis on 1% agarose gel buffered with 1XTAE, stained with ethydium bromide and photographed under ultraviolet light. Molecular weights were estimated using a Promega 1kb DNA Ladder.

Data analysis

RAPD are dominant markers. Amplified fragments were scored for the presence (1) or absence (0) of homologous bands, and were analyzed with TFPGA 1.3 (Miller 1997) genetic analysis sofware. Nei's gene diversity, the percentage of polymorphic loci and Nei's unbiased genetic distance were calculated. Faint bands were not recorded for analysis. Dendrograms were constructed using the UPGMA method based on Nei's (1972) genetic distances.

Tab. 1 Nucleotide sequences of the RAPD primers used to amplify genomic DNA from *Pinus sylvestris* populations

Primer identification	Nucleotide sequence (5'-3')	Amplification
PIN 1	CAGGCCCTTC	Moderate
PIN 2	GAAACGGGTG	Good
PIN 3	CAATCGCCGT	Good
PIN 4	AGGTGACCGT	Poor
PIN 5	CAAACGTCGG	Absent
PIN 6	GAGACGCACA	Absent
PIN 7	CACAGACACC	Good
PIN 8	GTAGCACTCC	Good
PIN 9	GTGTCTCAGG	Poor
PIN 10	TCCCGCCTAC	Poor
PIN 11	ACGACGTAGG	Absent
PIN 12	GAGGGCCTGA	Absent

Results and discussion

Evaluation of primers

Because RAPD PCR is sensitive to reaction parameters, we optimized the reaction conditions, as described in the Materials and Methods section. Five of the 12 primers generated strong amplification products. They were CAGGCCCTTC, GAAACGGGTG, CAATCGCCGT, CACAGACACC, GTAGCACTCC. Moderate and poor amplification products (primers: CAGGCCCTTC, AGGTGACCGT, GTGTCTCAGG, TCCCGCCTAC) were also included for analysis.

Genetic variability

All estimates of genetic variation based on RAPD were very low in *P. sylvestris.* The 8 RAPD primers produced a total of 208 bands in the 15 individuals. From the 16 loci surveyed 9 were polymorphic in the populations. Genetic identity within populations was 96.25% in Mohos,

Tab. 2. Nei's (1972/1978) genetic identities and distances for *Pinus sylvestris* populations (1-Mohos population; 2-Luci population; 3-Sumuleu population)

Populations compared	Distance	Identity	Unbiased	
			Distance	Identity
1 versus 2	0.1532	0.8580	0.1443	0.8656
1 versus 3	0.3045	0.7375	0.2836	0.7531
2 versus 3	0.2701	0.7633	0.2476	0.7807

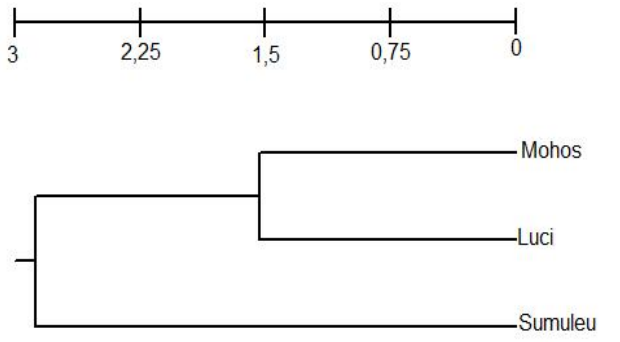

Fig. 1. UPGMA analysis for Nei's genetic distances (1-Mohos population; 2-Luci population; 3-Sumuleu population)

86.25% in Luci and 77.50% in Sumuleu Mountain. Genetic variability between populations was evaluated with Nei's method.

A primary objective of conservation genetics is to estimate the level and distribution of genetic variation in endangered species (Forrest, 1994). Knowledge of the level and distribution of genetic variation is a prerequisite for the establishment of effective and efficient conservation practices.

The generally low level of genetic variation of *Pinus sylvestris* results from its evolutionary processes. In papers concerning pine species that have been studied, it was hypothesized that a historical genetic bottleneck during the glacial episodes of the Holocene was the main reason for low polymorphism in this species (Eriksson, 1998; Mehes *et al*., 2007). The present populations form Mohos and Luci seem to be the descendants that survived the continental glaciation (Banarescu *et al*., 1973). Even in the absence of severe and prolonged bottlenecks, the small population size of *Pinus sylvestris* may have resulted in low genetic diversity, because of random genetic drift, and reduced gene flow between populations. The Mohos peat bog present low habitat quality, which increase the risk of gene diversity loss. Given the limited number of individuals of *Pinus sylvestris* forma *turfosa* in Mohos peat bog, it is necessary to protect all the existing individuals *in situ* in order to preserve as much genetic variation as possible. Between the two existing populations sometimes existed genetic flow, but in the last 10.000 years as a result of the habitat fragmentation leads to isolation of the populations. Nei's unbiased genetic identity is 0.86 between the two populations, and the intrapopulational genetic identity is 86.25% in Luci population, which means a total identity with the Mohos population. The 10% genetic variability loss in Mohos population in the last 10.000 years is a result of the reduced effective population size.

Higher intrapopulational genetic variability in Sumuleu population, can be explained by plantation of new *Pinus sylvestris* populations in the Ciuc Basin (as ornamental plants in the city's parks or in villages), which probably increased genetic diversity through wind pollination.

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