Wiss. Mitt. Niederösterr. Landesmuseum	12	53-62	St. Pölten 1999

DNA polymorphisms in Austrian and Dalmatian black pine

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Abstract

The systematic status of Dalmatian black pine is still unexplained. This black pine is usually considered as subspecies but sometimes also as a separate species. Austrian black pine from the Botanical Garden Zagreb, Dalmatian black pine from the mount of Biokovo and Dalmatian black pine from the island of Hvar were used in this study. Two methods of plant molecular systematics have been applied in this work: restriction fragment length polymorphisms of chloroplast DNA (RFLP cpDNA) and random amplified polymorphic DNA (RAPD). The results of RFLP analysis of chloroplast DNA have shown that there are no differences in restriction fragments among all investigated plants: As chloroplast DNA is evolutionary conservative DNA, conclusion has been drawn that all examined plants probably belong to the same species. The RAPD method which is the most appropriate for plant systematic at the species level and below, has shown DNA polymorphisms between and within the investigated populations, distinctly separated Austrian and Dalmatian black pine. The subspecies level for Dalmatian black pine was strongly supported by these methods. RAPD method demonstrated higher similarity between Austrian black pine with Dalmatian black pine from Biokovo than Austrian black pine with Dalmatian black pine from the island of Hvar and reminds us of an earlier hypothesis to say that the black pine from Biokovo represents an intermediate population between Austrian and Dalmatian black pine.

Zusammenfassung

Der systematische Status der dalmatinischen Schwarzkiefer ist noch immer ungeklärt. Sie wird gewöhnlich als eine Unterart betrachtet, manchmal aber auch als selbständige Art *Pinus dalmatica* Vis.. Die österreichische Schwarzkiefer aus dem Botanischen Garten Zagreb, die dalmatinische Schwarzkiefer vom Gebirge Biokovo und die dalmatinische Schwarzkiefer von der Insel Hvar wurden bei dieser Forschung betrachtet. Es wurden zwei Methoden der Molekularsystematik angewendet: restriction fragment lenght polymorphisms of chloroplast DNA (RFLP cpDNA) und random amplified polymorphic DNA (RAPD). Die Ergebnisse der RFLP Analyse der Chloroplast-DNA haben gezeigt, daß keine Unterschiede zwi-

schen den Restriktionsfragmenten der untersuchten Pflanzen bestehen. Weil Chloroplast-DNA ein evolutionär konservatives Molekül ist, konnte man schließen, daß alle untersuchten Pflanzen wahrscheinlich derselben Art angehören. Die RAPD Methode, die für die Pflanzensystematik auf dem Niveau der Art, wie auch auf den niedrigeren systematischen Niveaus, die geeignetste ist, hat einen Polymorphismus zwischen und innerhalb der untersuchten Populationen gezeigt und trennt deutlich die österreichische und die dalmatinische Schwarzkiefer. Durch diese Methode wurde das Niveau der Unterart für die dalmatinische Schwarzkiefer klar unterstützt. Die RAPD Methode hat eine größere Ähnlichkeit zwischen der österreichischen Schwarzkiefer und der dalmatinischen Schwarzkiefer vom Gebirge Biokovo gezeigt, als zwischen der österreichischen Schwarzkiefer und der dalmatinischen Schwarzkiefer von der Insel Hvar. Dies hat uns an eine frühere Hypothese erinnert, nach der die Schwarzkiefer vom Gebirge Biokovo eine Übergangspopulation zwischen der österreichischen und der dalmatinischen Schwarzkiefer sei.

Key words: black pine, CpDNA, DNA polymorphisms, *Pinus nigra,* RAPD, RFLP, molecular systematics

INTRODUCTION AND METHODS

Introduction

European black pine (*Pinus nigra* Arnold) has a natural distribution in southern Europe, extending from Spain to Turkey (JALAS & SUOMINEN 1988). This very variable species contains numerous subspecies, varieties and forms, and its systematics is still unexplained and is very dependent on the various authors point of view (GAUSSEN ET AL. 1964, VIDAKOVIĆ 1982). Variability of morphological and anatomical characteristics of this black pine is in concordance with discontinuous distribution of this species. Dalmatian black pine (*Pinus nigra* Arnold subsp. *dalmatica* (Vis.) Franco) is endemic on the Dalmatian island of Brač, Hvar, Korčula, peninsula Pelješac and the mount Biokovo. Roberto Visiani was the first one who described this black pine (VISIANI 1842). This author considers Dalmatian black pine as a separate species *Pinus dalmatica* Vis. Many authors used in their investigations only morphological and anatomical traits which are very variable and strongly dependent on the environmental condition. The methods of plant molecular systematics assay genotypic variation directly and obtain characters of more fundamental nature, free of nonheritable environmental perturbations that obscure true genetic relationships. The methods of plant molecular systematics could be a valuable tool for realising the systematics of *Pinus nigra* complex. This study is a beginning of the more intensive molecular systematics studies of Dalmatian black pine and the whole European black pine complex. In this preliminary study we used two methods of plant molecular systematics and only three populations of black pine.

Plant material

Bulked seed samples of Dalmatian black pine were collected from two natural populations, the mount Biokovo and the island of Hvar (Fig. 1). Bulked seed samples of Austrian black pine were collected from the Botanical Garden of the Faculty of Science, Zagreb, Croatia. Composite needle samples were harvested from seedlings of each populations, and used for chloroplast and total DNA extraction.

Chloroplast DNA isolation and analyses

CpDNA was isolated following a method described by SZMIDT & WANG (1993.). CpDNA samples from each population were digested with three restriction enzymes: *Bam* HI, *Eco*RI and *Hind* III. Electrophoresis of restriction fragments was carried out in 0.8 % agarose gel, for 16 hours, at 2.7 V/cm, in TAE buffer pH 8.0. The gels were stained with ethidium bromide, visualised under UV light, and photographed using Polaroid camera. After denaturation and neutralisation, the separated CpDNA fragments were transferred to nylon membrane (SOUTHERN 1979) and hybridised with ^{32}P labelled total spinach Cp DNA (FEINBERG & VOGELSTEIN 1983, HERRMANN 1981, SAKATA & LESTER 1994). Hybridisation was carried out at 65° C in the solution of 6xSSC, 2xDenhardt's solution, 2.0 mM EDTA, pH 8.0, 0.2% SDS (SAMBROOK ET AL. 1989). The membrane was washed in 0.1 x SSC, 0.1% SDS, 3 x 15 min at room temperature and 2 x 15 min at 50 °C and exposed to X-ray film.

Total DNA isolation and RAPD procedure

Total DNA was isolated following a method described by SZMIDT & WANG (1993). RAPD PCR was performed in a volume of 50 ml containing 1 x PCR buffer, 2.5 mM $MgCl_2$, 5 pmol primer, 0.2 mM of each dNTPs, 50ng DNA and 1 unit of Amplitaq DNA polymerase (Perkin Elmer Cetus). Each amplification was performed using a single primer: $GATA_4$, OPB12, OPB14, OPB15 and OPB16 (Operon Technologies). The amplification condition were as follows: the first step of 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C (50 °C for $GATA_4$ primer) and 2 min at 72 °C. Amplification was carried out using the Perkin Elmer Cetus thermal cycler. Amplification products were analysed by electrophoresis on 1.4% agarose gels and detected by staining with ethidium bromide and photographed using Polaroid camera.

Statistics

CpDNA and RAPD bands were scored for molecular weight. CpDNA patterns were identical between all black pine populations and statistics was unnecessary. RAPD bands between populations were scored as present or absent. For the estimation of genetic similarities between compared populations of black pine two algorithms were used: the Dice (DICE 1945) and the Simple Matching (SOKAL & MICHENER 1958). These algorithms calculate the degree of affinities on the bases of shared characters as a proportion of the total characters. The RAPD band intensity also was scored as: 0 = no band, 1 = faint band, 2 = medium and 3 = very bright band (ADAMS & DEMEKE 1993) and similarity measures were computed using absolute character state differences by Manhattan metric (GOWER 1971). The NT-SYS-pc package of computer programs, version 1.60, (ROHLF 1990) was used for computing.

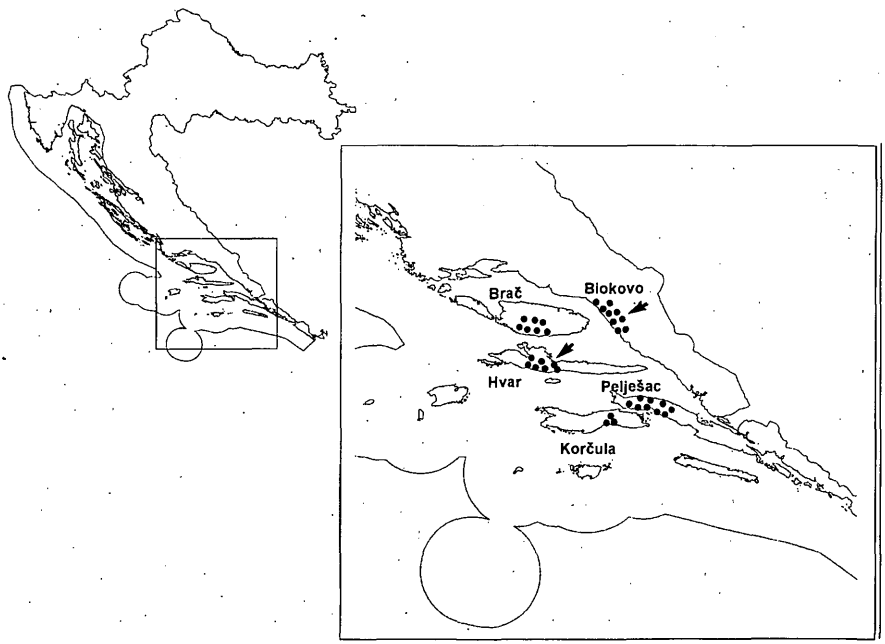

Fig. 1: Distribution of Dalmatian black pine *Pinus nigra* Arnold subsp. *dalmatica* (Vis) Franco in Croatia. Populations which were used in this study are marked with arrows.

RESULTS

RFLP CpDNA

The pure chloroplast DNA could not be isolated even after many attempts of isolation. Chloroplast DNA was always contaminated with nuclear DNA, which gave strong background to the CpDNA bands. Because of that a heterologous hybridisation of pines CpDNA with ^{32}P-labelled total pure CpDNA of spinach was used. After isolation of pines CpDNA, digestion with restriction enzymes *BamHI*, *EcoRI*, and *HindIII*, agarose gel electrophoresis, hybridisation and autoradiography some restriction fragments in size between 1.0 to 12.2 kb could have been detected. The restriction fragments were the same in all investigated populations of black pine (Fig. 2).

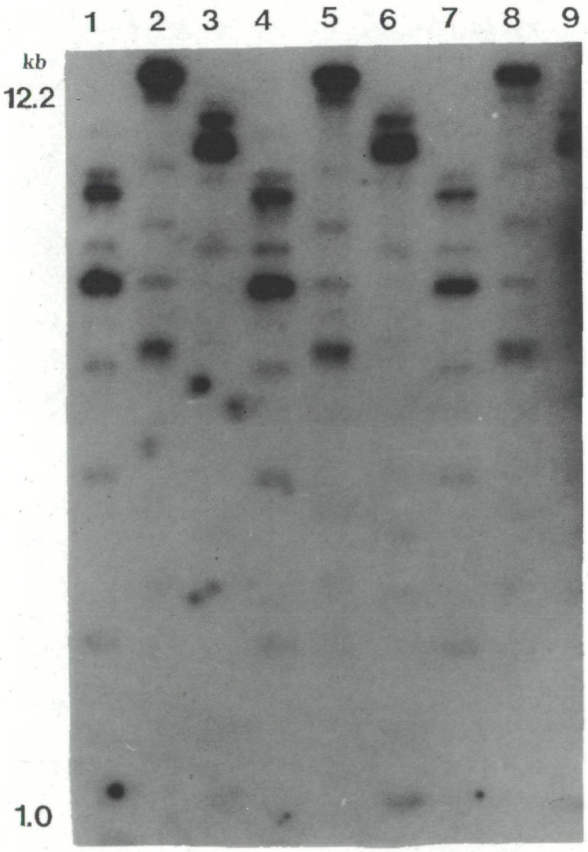

Fig. 2. Restriction fragment length polymorphisms of chloroplast DNA (RFLP CpDNA) of three different populations of European black pine.
Lines 1, 2, 3. Austrian black pine / Bam HI, Eco RI, Hind III;
Lines 4, 5, 6. Dalmatian black pine (Hvar) / *Bam* HI, *Eco* RI, *Hind* III;
Lines 7, 8, 9. Dalmatian black pine (Biokovo) / *Bam* HI, *Eco* RI, *Hind* III

RAPD

As cpDNA is evolutionary very conservative DNA molecule and rare polymorphic below species level, RAPD method was used because it is more sensitive than RFLP cpDNA. Twenty-one primers were tested but five were found informative: $GATA_4$, OB-12, OB-14, OB-15 and OB-16. These primers were revealed separate RAPD polymorphisms for each investigated population. Five primers generated a total of 90 scoriable RAPD bands (Fig. 3).

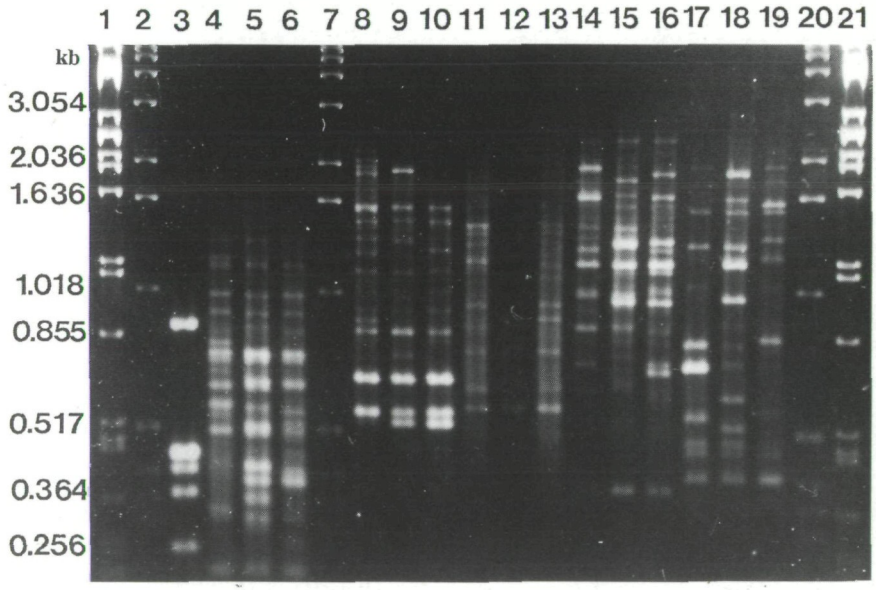

Fig. 3: Random amplified polymorphic DNA (RAPD) of three different populations of European black pine. Lines 1, 2, 3. DNA Size Standards: λ DNA / Pst I, 1 Kb DNA Ladder, pWH802; Lines 4, 5, 6. GATA4: Austrian black pine, Dalmatian black pine (Hvar), Dalmatian black pine (Biokovo); Line 7. DNA Size Standard: 1 Kb Ladder; Lines 8, 9, 10. OPB12: Austrian black pine, Dalmatian black pine (Hvar), Dalmatian black pine (Biokovo); Lines 11, 12, 13. OPB14: Austrian black pine, Dalmatian black pine (Hvar), Dalmatian black pine (Biokovo); Lines 14, 15, 16. OPB15: Austrian black pine, Dalmatian black pine (Hvar), Dalmatian black pine (Biokovo); Lines 17, 18, 19. OPB16: Austrian black pine, Dalmatian black pine (Hvar), Dalmatian black pine (Biokovo); Line 20, 21. DNA Size Standards: 1 Kb DNA Ladder, λ DNA / Pst I

Statistics

The Dice and Simple matching matrices of similarity is based on the RAPD markers are shown in Table 1. Manhattan metric that measures differences are shown in Table 2. These results separate Dalmatian black pine populations from Austrian black pine population and also demonstrate higher similarity between Austrian black pine and Dalmatian black pine from Biokovo than Austrian black pine and Dalmatian black pine from the island of Hvar.

Table 1. Dice similarity (lower half) and simple matching (upper half) matrices for the populations of black pine based on the RAPD markers.

Populations	**1**	**2**	**3**
1. *Pinus nigra* subsp. *nigra*	*	0.49	0.51
3. *Pinus nigra* subsp. *dalmatica* (Hvar)	0.58	*	0.60
3. *Pinus nigra* subsp. *dalmatica* (Biokovo)	0.60	0.69	*

Table 2. Manhattan metric matrices for the populations of black pine based on the RAPD markers.

Populations	**1**	**2**	**3**
1. *Pinus nigra* subsp. *nigra*	*		
3. *Pinus nigra* subsp. *dalmatica* (Hvar)	0.99	*	
3. *Pinus nigra* subsp. *dalmatica* (Biokovo)	0.90	0.65	*

DISCUSSION

Black pine has phenotypic characteristics that are strongly dependent on the environment conditions. The systematics of this complex is based exclusively or mostly on phenotypic characters and probably this is a reason why many systematic problems are still unexplained. Dalmatian black pine with its morphological and anatomical characteristics essentially differs from other species of black pine complex and was even considered as a separate species: *Pinus dalmatica* Vis.. For decades, a goal of many systematics has been to penetrate the barrier of the phenotype and obtain characters of more fundamental nature. Recombinant DNA technology and methods of molecular systematics has permitted the systematists to

solve these problems by assaying genotypic variation directly (DOYLE 1993).

Our intention was using the technics of molecular systematics to establish the genetic relationships between Dalmatian black pine and Austrian black pine and to determine systematic status of Dalmatian black pine.

RFLP cpDNA is the most used method in plant molecular systematics and very good for a determination of species of genus *Pinus* L. (STRAUSS & DOERKSEN 1990, WANG & SZMIDT 1993). The RFLP cpDNA showed that there are no differences in BamHI, EcoRI and Hind III restrictions fragments among examined populations and that they probable belong to the same species. As chloroplast DNA is evolutionary conservative DNA can be used as a hybridisation probe for the evolutionary distant groups. Because of that when we couldn't isolate enough pure cpDNA of black pine we used total high pure cpDNA of spinach as hybridisation probe. In this way we obtain stronger signals of cpDNAs fragments of black pine. Because of phylogenetical distance between hybridisation probe and investigated cpDNA we couldn't obtain all restriction fragments and some ones were very weak.

RAPD method is one of the variations of PCR that use single 10-base oligonucleotide primer to amplify DNA. Polymorphisms of RAPD DNA originates from point mutations, insertions, deletions and inversions (WILLIAMS ET AL. 1990). The most of systematics studies that use RAPD methods are on the level of species and below (DEMEKE & ADAMS 1994). Using RAPD and RFLP cpDNA methods we tried to assay the systematic categories on the level of species and below. RAPD DNA profiles are translated into two types of statistics data: as binomial (Dice and Simple matching coefficient) and meristic (Manhattan metric). Dice algorithms consider only shared presence of DNA band as a measure of similarity, but not absence of DNA band. Simple matching algorithms consider shared presence and absence DNA bands as an indication of similarity. For Manhattan metric RAPD bands because of their different origin, are scored as: 0 = no band; 1 = faint band; 2 = medium; 3 = bright band (ADAMS & DEMEKE 1993). This translation of RAPD DNA bands makes possible better comparison among investigated populations.

The RAPD study demonstrate higher genetic similarity between Austrian black pine and Dalmatian black pine from Biokovo than Austrian black pine and Dalmatian black pine from the island of Hvar. It reminds us of an earlier hypothesis according to which the black pine from Biokovo represents a transitive population from Austrian towards Dalmatian black pine.

As we know that RFLP cpDNA is a high value tool for plant systematics at the species level and RAPD at the level of the same species and below, we can draw a conclusion that all examined plants in our assay belong to the same species and that Dalmatian and Austrian black pine are distinctly separated as two subspecies. But we have to take these conclusions with same doubt because this study is limited by small number of individuals of black pine, three restriction enzymes, and only twenty RAPD primers.

This assay point to the further *Pinus nigra* Arnold complex investigation which

should be proceeded by using molecular systematics methods, more populations of Dalmatian and other black pines, more restriction enzymes, cpDNA hybridisation probes of pines, more RAPD primers. The future studies should also include traditional systematics methods. In such a way genetic relationships and systematic status of Dalmatian black pine and other members of *Pinus nigra* complex will be able to be solved.

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