Estimation of the Introgression Level in *Populus nigra* L. Populations by Means of Isozyme Gene Markers

By V. BENETKA, J. MOTTL, K. VACKOVÁ, M. POSPÍŠKOVÁ and M. DUBSKÝ

Research Institute of Ornamental Gardening Průhonice, 252 43 Průhonice, Czech Republic

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Summary

Three enzyme systems (6-PGD, LAP, GPI) were tested in the progeny of a controlled crossing of *Populus* x *canadensis* x *Populus nigra* with the object of distinguishing hybrids in a population of *P. nigra*. Segregation ratios of 1:1 for 6-PGD and LAP were determined among phenotypes corresponding to *P. nigra* (homozygote) and *P.* x *canadensis* (heterozygote). The segregation ratio for GPI was 3:1 in favour of the homozygote. No linkage was found between the polymorphic loci of 6-PGD and LAP. Experimental results proved that it is possible to identify hybrids of the *P.* x *canadensis* x *P. nigra* type from spontaneous populations of *P. nigra*. These results enabled analyses of three populations of *P. nigra*. In the generative progeny of the female trees of *P. nigra* no hybrids were ascertained. Hybrids morphologically different from *P. nigra* were identified in a population of two-years-old seedlings. In a population of trees 10 to 18 years of age 9.7% were hybrids with *P.* x *canadensis* yet not different from *P. nigra* morphologically. The extent of introgression in the progeny of female *P. nigra* is discussed.

Key words: Populus nigra L., hybrid poplars, populations, introgression, isozyme analysis.

Introduction

Spontaneous interspecific hybridization in the genus *Populus* has been observed from the beginning of the introduction of the American poplar *Populus deltoides* MARSH. at the turn of 17th and 18th centuries in Europe, both within section *Aigeiros* DUBY and between sections *Aigeiros* and *Tacamahaca* SPACH (HESMER, 1951). The main introduced species in the Czech Republic are *Populus* x *canadensis* MOENCH (hybrid between *Populus deltoides* and *P. nigra*); *Populus* x *berolinensis* DIPPEL (hybrid between *Populus laurifolia* LEDEB. and *Populus nigra* var. *italica* DUROI) and later on also *Populus trichocarpa* TORR. et GRAY (MOTTL, 1989).

Gene exchanges between the wild and the cultivated gene pool may also occur spontaneously (CAGELLI and LEFÉVRE, 1995). As found by BISOFFI et al. (1987) spontaneous hybridization between *P. nigra* and the female clones of *P.* x *canadensis* makes the selection of genetically pure individuals of *P. nigra* more difficult.

The incorporation of genes of one species into the gene pool of a second species with the aid of hybridization and backcrossing was designated by ANDERSON and HUBRICHT (1938) as introgression.

The progeny of interspecific hybridization *P. nigra* x *P.* x *canadensis* is so similar in morphological traits to *P. nigra* species, that the identification of the progeny by means of morphological traits is problematic. Therefore, use of biochemical markers analysis may prove suitable for the differentiation. These markers, especially isozymes have been used for the study of genetic composition of various populations. Isozyme gene markers (allozymes) are useful up to now the genetic markers, which combine the estimation of genetic variability with the characterization of plant populations (PÉREZ DE LA VEGA, 1993).

The first authors to use isozymes for clone identification in the genus *Populus* were GUZINA (1978), BERGMANN (1981), CHELIAK and PITTEL (1984). Genetic analysis of enzymes in 4 species (*P. deltoides, P.* x *canadensis, P. nigra* and *P. maximowiczii*) was elaborated by RAJORA (1986, 1989, 1990) and RAJORA and ZSUFFA (1990) and in intra- and interspecific full sib families of the *Tacamahaca* section and intersectional hybrids (MÜLLER-STARCK, 1992).

While it is possible to differentiate between pure parent species and hybrid plants of the first generation with individual biochemical markers, further generations of hybrids and B_1-hybrids from backcrossing cannot be easily assessed. Also in this type of hybrids, segregation will occur according to the principles of MENDELian heredity. When more enzyme systems are combined the probability of distinguishing between such hybrid individuals increases (JANSSEN, 1997; HEINZE, 1997).

Even though it is suggested that the species *P. nigra* cannot be propagated spontaneously due to loss of natural stands – river flood plains – (FRISON et al., 1995) we observed, that poplars can be propagated even in alternative stands, which are temporarily without vegetation cover and which have good moisture conditions.

The objective of this research was to identify how much genetic structures of such populations are influenced by introgression from *P.* x *canadensis*.

Material

A. Test population

Segregation ratios were tested on seedlings from the controlled crossing of *Populus* x *canadensis* and *P. nigra*. The *P.* x *canadensis* clone 'Marylandica', the most distributed tree in older plantings in the Czech Republic, was chosen as the mother plant. Three plus trees of black poplars from two different areas (the Morava and the Elbe river basins) – (*Table 1*) were selected as the male plants. The morphological and isozymic traits of the male trees corresponded to the pure species *P. nigra* (TUROK et al., 1996).

Table 1. – Parental plus trees of *P. nigra*.

Number of plus trees	Sex	Latitude	Longitude	Years	Basin
880011	f	04849N	01656E	40	--
880012	f	04849N	01656E	40	--
880044	m	04936N	01720E	140	Morava
880046	m	04936N	01720E	80	Morava
880060	f	05001N	01517E	140	Elbe
880061	m	05001N	01517E	140	Elbe

B. Natural population

1) *Progenies of three female trees:* Seeds from mass pollination of three plus trees of *Populus nigra* were harvested, sown and after raising seedlings, 450 individuals were outplanted near the Morava river bank in 1994.

The female plus trees *P. nigra,* No. *880011 and 880012* (*Table 1*) are 40 years old. They grow in the wind break stands in South Moravia. Trees of the species *P.* x *canadensis,* clone 'Gerlica', grow also in this wind break. Other male clones 'Brabantica' and 'Robusta' grow 4 km to 5 km from there.

The female plus tree *P. nigra* No. *880060* was around 140 years old. It is a solitary tree growing near the middle of the Elbe flow. A male plus tree *P. nigra* No. *880061* of the same age is quite near. No individual of *P.* x *canadensis* has been observed nearby. This species can be found a few kilometers from there.

2) *Populations of two-year-old seedlings:* The study area (50° 10' N; 14° 43' E) is about 15 m x 30 m. The place is very waterlogged. Soil cover from a near-by gravel-pit was placed on this area. A dense cover of poplar seedlings has grown on the bare soil. Female plants of *P. nigra* 60 to 80 years old grow in the surroundings of the locality at a distance of around 500 m. There are also male plants which could serve as a pollen source. Some hybrids of *P.* x *canadensis* grow near the study area. In the wider surroundings, other trees of the genus *P.* x *canadensis* exist.

3) *Tree populations 10 to 18 years of age:* The study area (50° 15' N; 14° 18' E) is a slope of a former sand-pit. The trees create a coherent and well balanced stand of about 100 individuals. Around 200 m from the study area, single trees of *P. nigra,* 60 to 80 years old and 4 trees 100 years of age can be found. These trees are there together with *P.* x *canadensis,* clone 'Marylandica', stands. A group of around 30 years old trees of *P.* x *canadensis,* for which the clones have not been precisely determined, exists approximately 100 m from that stand.

Methods

Isozyme analysis

Tissues of actively growing root tips from rooted cuttings were used for gel electrophoresis. Branches for cuttings were collected from January to March and were rooted in a greenhouse. Five to ten root tips were homogenized in 50 µl to 100 µl of extraction buffer (RAJORA, 1989). The extract was absorbed onto 3 mm x 9 mm filter-papers which were inserted into horizontal starch gel slabs.

Four enzyme systems were assayed by horizontal starch gel electrophoresis using three different buffer systems (*Table 2*).

Table 2. – Buffer systems, electrophoretic conditions and enzymes.

Buffer system	Voltage	Enzyme			
		Name EC No.	Abbr.	Quaternary structure	Loci*
A) morpholine-citrate (Clayton and Tretiak 1972)	230 V	6-phosphogluconate dehydrogenase 1.1.1.44	6-PGD	dimer	5
		aconitase 4.2.1.3	ACO	monomer	3
B) lithium-borate (Selander et al. 1971)	290 V	leucine aminopeptidase 3.4.11.1	LAP	monomer	2
C) tris-citrate (Siciliano and Shaw 1976)	190 V	glucosephosphate isomerase 5.3.1.9	GPI	dimer	2

*) RAJORA (1986)

Starch gels (12%) were prepared from potato starch (Sigma S 5651, St. Louis, MO, USA) (buffer A and C) and from hydrolyzed potato starch (Škrobárna Brno, the Czech Republic) (buffer B). After electrophoresis was completed, the gels were sliced horizontally. Gel slices were stained with specific histochemical stain assays (WENDEL and WEEDEN, 1989, modified; and VALLEJOS, 1983).

Hybridization was performed on cut branches with flower buds in a greenhouse. Similarly pollen was taken from the speeded up branches, which were specially isolated. Inflorescence were isolated by semiparchment bags. Pollen grains were transferred by air flow in little rubber balloons through the wall of a perforated insulator.

Results

a) Isozyme analysis

Spectra of 4 enzyme systems (6-PGD, LAP, GPI and ACO) achieved by analysis of individuals of *P. nigra, P.* x *canadensis* and *P.* x *berolinensis* are presented in *figure 1.* Spectra 6-PGD, LAP and GPI serve for the differentiation of *P. nigra* from *P.* x *canadensis* and ACO for the differentiation of *P.* x *berolinensis* from *P. nigra* and *P.* x *canadensis.* We chose those enzyme systems based on the results of RAJORA (1986). We confirmed these his results by analysing 35 individuals with the morphological characteristics of *P. nigra* (TUROK et al., 1996). Samples of *P.* x *canadensis* and *P. deltoides* were taken from clone collections. We carried out 19 combinations of crossings with 17 individuals of *P. nigra* and in their offsprings we conducted an isozyme analysis.

6-PGD

Enzyme system 6-PGD has 3 monomorphic zones of activity genetically coded at loci 6-Pgd-1, 6-Pgd-2 and 6-Pgd-3 (the third one is missing in *P.* x *berolinensis*) and 2 polymorphic zones (loci 6-Pgd-4 and 6-Pgd-5), which are used to distinguish between *P.* x *canadensis* on the one side and *P. nigra* and *P.* x *berolinensis* on the other. Products of the genes at 6-Pgd-4 and 6-Pgd-5 loci are detected on the gel as 3 bands in the case of *P. nigra,* as 5 bands in the case of *P.* x *canadensis* and as 2 bands in the case of *P.* x *berolinensis.*

LAP

Two zones of leucine aminopeptidase activity (loci Lap-1 and Lap-2) were observed in conformity with RAJORA (1989, 1990) and MÜLLER-STARCK (1992). Product of the gene at locus Lap-1 is detected as 1 band in zymograms of *P. nigra* and *P.* x *berolinensis* (homozygous type) and as a double band in zymograms of *P.* x *canadensis* (heterozygous type). Gene at Lap-2 is not expressed in *P. nigra* and *P.* x *berolinensis,* while *P.* x *canadensis* individuals were single-banded monomorphic in this locus. By using LAP *P.* x *canadensis* can be distinguished from both *P. nigra* and *P.* x *berolinensis* too.

GPI

Enzyme system GPI produces 2 zones of activity genetically coded in loci Gpi-1 and Gpi-2. The first zone is monomorphic, the second polymorphic zone consist of 3 to 5 bands, *P.* x *canadensis* is characterised by a higher number of bands and slower mobility towards anode (+), which in fact can be used for distinguishing it from individuals of *P. nigra* and *P.* x *berolinensis.*

ACO

Three zones of aconitase activity were observed. Two of them (Aco-1 and Aco-2) correspondent to those described by RAJORA (1989, 1990) and MÜLLER-STARCK (1992). The third one (Aco-3) we found only in samples of *P.* x *berolinensis.* Aco-3 (with 2

bands) thus appears to be suitable markers for distinguishing of *P.* x *berolinensis* from *P. nigra* and *P.* x *canadensis.*

b) Segregation ratios of phenotypes in individual enzyme systems after test crossing and the probability of hybrid detection

Segregation ratios were observed in individual enzyme systems (6-PGD, LAP, GPI) in three half-sib seedling sets. A schematic illustration of the species *P. nigra* and *P.* x *canadensis* alleles in individual enzyme systems is demonstrated in *figure 1.* Homozygous (see *P. nigra*) and heterozygous (see *P.* x *canadensis*) phenotypes in individual experimental variants were determined according to this scheme. Segregation ratios of three enzyme systems in three test populations ('Marylandica' x 880044; 'Marylandica' x 880046 and 'Marylandica' x 880061) are demonstrated in *table 3* and *figure 2.*

Table 3. – Segregation ratios of homozygote and heterozygote phenotypes in three enzyme systems (6-PGD, LAP and GPI) of three progenies from controlled pollination *P.* x *canadensis* x *P. nigra.*

Locus	M x 880044 - : x	M x 880046 - : x	M x 880061 - : x	Σ - : x	Found ratios	χ^2 value
6–Pgd –4&5	26 : 33	5 : 5	10 : 8	41 : 46	1 : 1	0,287
Lap–1	25 : 26	5 : 5	9 : 8	39 : 39	1 : 1	0,000
Gpi–2	15 : 4	6 : 2	8 : 5	29 : 11	3 : 1	0,133

88044, 880046, 880061 – plus trees *Populus nigra;* M – 'Marylandica'
– homozygote phenotypes (see *P. nigra* at *Figure 1*)
x heterozygote phenotypes (see *P.* x *canadensis* at *Figure 1*)

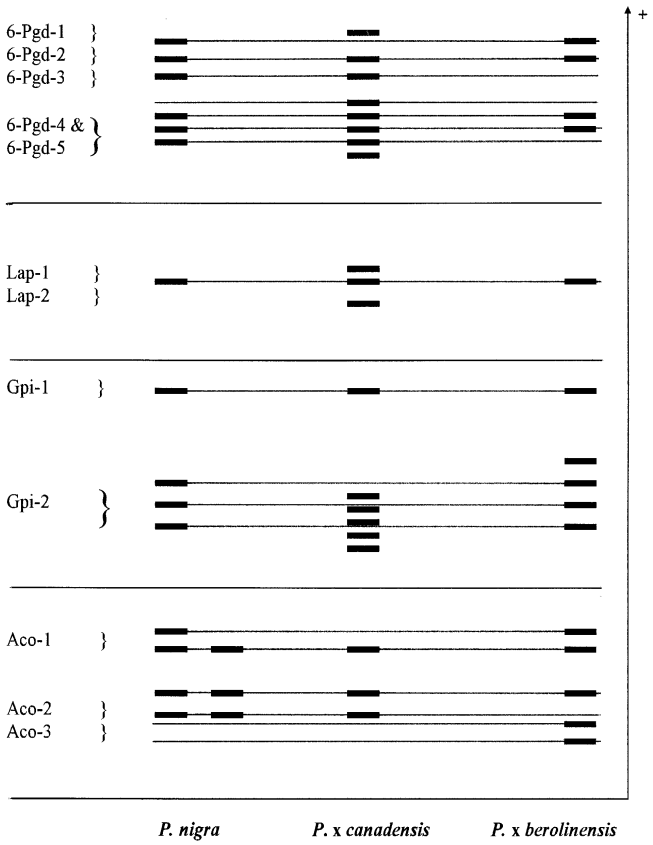

Figure 1. – Schematic representation of the banding patterns for 6-PGD, LAP, GPI and ACO loci shown by three *Populus* species.

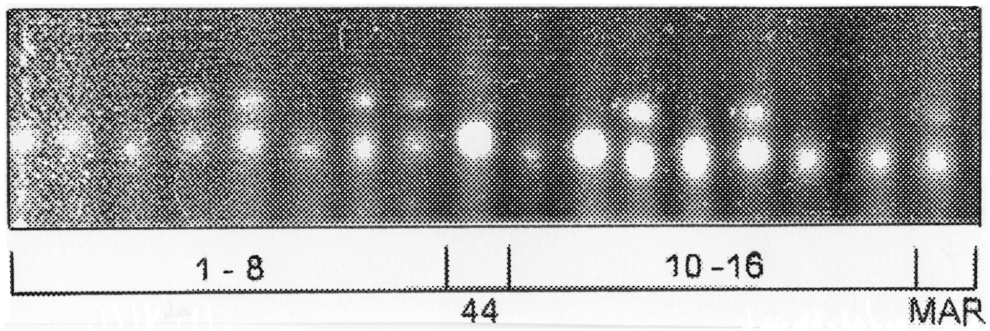

Figure 2a. – Zymograms of Lap-1 of 15 siblings from the controlled pollination *P.* x *canadensis* x *P. nigra.* At runs 4, 5, 7, 8, 12, 14 there are heterozygotic phenotypes; at runs 1, 2, 3, 6, 10, 11, 13, 15, 16 are homozygotic phenotypes. Parents are at run 9 (*P. nigra* – plus trees 880044) and run 17 (*P.* x *canadensis* cv. Marylandica – MAR).

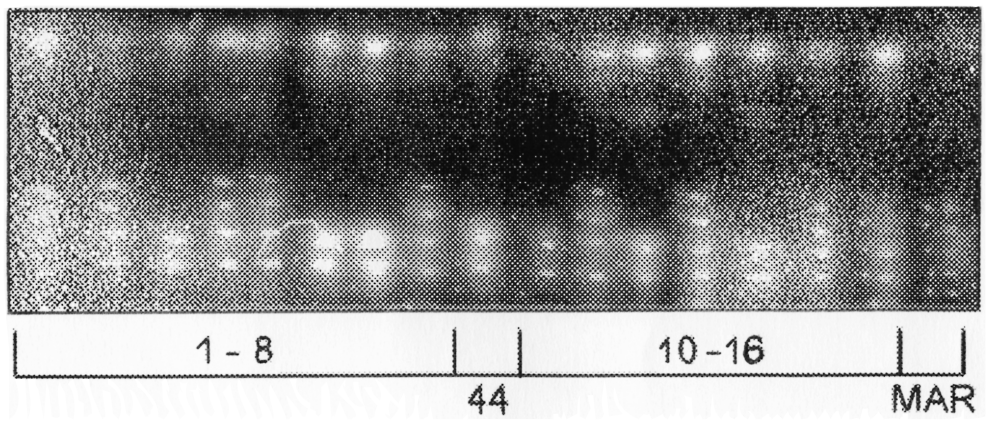

Figure 2b. – Zymograms of 6-Pgd-4 and 5. At runs 1, 2, 4, 5, 8, 11, 13, 15, 16 there are heterozygotic phenotypes; at runs 3, 6, 7, 10, 12, 14 are homozygotic phenotypes. Parents are at run 9 (*P. nigra* – plus trees 880044) and run 17 (*P.* x *canadensis* cv. Marylandica – MAR).

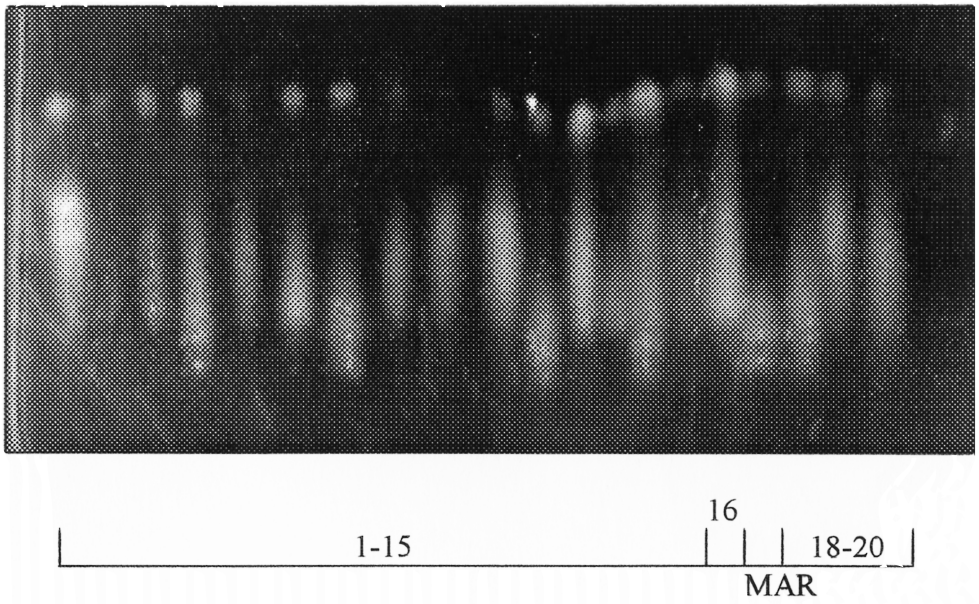

Figure 2c. – Zymograms of Gpi-2. At runs 4, 7, 11, 14, 18 there are heterozygotic phenotypes; at runs 1, 2, 3, 5, 6, 8, 9, 10, 12, 13, 15, 19, 20 are homozygotic phenotypes. Parents are at run 16 (*P. nigra* – plus trees 880046) and run 17 (*P.* x *canadensis* cv. Marylandica – MAR).

The given values are mostly the results of two to four successful analyses. While in the loci 6-Pgd-4 and 5 and Lap-1 the coincidence of segregation ratios with the expected ratio 1:1 was proved, in Gpi-2 the ratio found was 3:1 in favour of homozygous phenotypes. The independent trait combination between 6-PGD and LAP is shown in *table 4.*

In case of the evaluation of a single hybrid progeny by three enzyme systems at the same time, when the segregation ratios in the individual enzyme systems are 1:1; 1:1; 3:1 and if we assume an independent gene combination, we obtain the probability P = 81.25% for hybrid phenotypes and 18.75% for the homozygotes.

c) Identification of hybrids in a test population

From the above-mentioned group 37 individuals were randomly selected. In these individuals analysis of all 3 enzyme systems (6-PGD, LAP and GPI) were carried out, resulting in the detection of 31 hybrids in the set of 37 individuals (*Table 5*) and only 6 individuals which appeared to be homozygous. This ratio corresponds to the assumed interpretation of 81.25 : 18.75.

d) Estimation of the introgression level in natural populations

1) *Progenies of three female trees:* The population consisted of 450 individuals, four years of age. According to morphological traits all the trees belonged to the species *Populus nigra.* The plants were sampled randomly for analysis. Thirty-three individuals were successfully analysed. Twenty-three individuals with P = 81% and 10 with P = 66% were not hybrids with *Populus* x *canadensis.*

2) *Population of two-year old seedlings:* Fifty-five plants were randomly selected for analysis. These plants corresponded to the species *Populus nigra* in their morphological traits (bark colour, leaf shape). Beside that 5 plants were purposely selected. They differed in bark colour (light brown shoot tips) and in one case in the angularity on the shoot tips, which was a typi-

Table 4. – Segregation ratios of dihybrid 6-PGD/LAP in a test population of a set 37 individuals from controlled pollination *P.* x *canadensis* x *P. nigra.*

Genotype	Phenotype	Observed frequency	Found ratios	χ^2 value
$P_1P_1L_1L_1$	- -	7	1	
$P_1P_1L_1L_2$	- x	10	1	
$P_1P_2L_1L_1$	x -	10	1	
$P_1P_2L_1L_2$	x x	10	1	0,7297

P = 6-PGD; L = LAP
P_1; L_1 = alleles of *Populus nigra*
P_2; L_2 = alleles of *Populus deltoides*
– homozygote phenotypes (see *P. nigra*)
x heterozygote phenotypes (see *P.* x *canadensis*)

Table 5. – Hybrids from the controlled pollination *P.* x *canadensis* x *P. nigra* identified using three enzyme systems (6-PGD, LAP, GPI).

n	6-PGD	LAP	GPI	Identified hybrid
6	-	-	-	-
4	x	x	x	x
6	x	x	-	x
8	x	-	-	x
4	-	x	x	x
1	-	-	x	x
6	-	x	-	x
2	x	-	x	x
Σ 37	20 : 17	20 : 17	11 : 26	31 : 6
	x : -	x : -	x : -	x : -

– homozygote phenotypes (see *P. nigra*)
x heterozygote phenotypes (see *P.* x *canadensis*)

cal trait of *Populus deltoides.* Fifty-seven individuals (from the total amount of 60) were successfully analysed. Fourty-five individuals with P = 81% and 8 with P = 66% were not hybrids with the species *Populus* x *canadensis* and 40 individuals were not crosses with *P.* x *berolinensis.* Four individuals appeared to be interspecific hybrids of *Populus nigra* with *Populus* x *canadensis.* However, the calculated percent of introgression (7%) was higher compared to the purposeful selection of hybrid individuals (*Tab. 6a*).

Table 6. – Hybrids identified in two natural populations of *P. nigra* using four enzyme systems 6-PGD, LAP, GPI, ACO;
a) population of two-year-old seedlings.

n	6-PGD	LAP	GPI	ACO	Identified hybrid	Pure *P. nigra* P (%)
2 *	x	x	-	-	x	
2 *	-	x	-	-	x	
1 *	-	-	-	-	-	81
31	-	-	-	-	-	81
13	-	-	-	o	-	81
8	-	o	-	-	-	66
3	o	o	o	o		
Σ60					**4**	**53**

* purposeful selection
o unsuccessful analyses

b) tree population – 10 to 18 years of age.

n	6-PGD	LAP	GPI	ACO	Identified hybrid	Pure *P. nigra* P (%)
25	-	-	-	-	-	81
3	-	-	o	o	-	75
1	-	x	x	x	x	
1	x	-	o	o	x	
1	x	x	-	-	x	
Σ 31					**3**	**28**

– homozygote phenotypes (see *P. nigra*)
x heterozygote phenotypes (see *P.* x *canadensis*)
o unsuccessful analyses

3) *Tree population – 10 to 18 years of age:* Individuals were found in a stand of 100 trees, visibly corresponding to the species *Populus nigra* in their morphological traits. Samples from 31 individuals were randomly selected for analyses. Twenty-five individuals with P = 81% and 3 with P = 75% were detected not to be hybrids with the species *Populus* x *canadensis.* The remaining 3 individuals were hybrids. Therefore, at least in 9.7% of individuals introgression from *P.* x *canadensis* was demonstrated. Again presence of hybrids with the species *Populus* x *berolinensis* was not detected (*Table 6b*).

Discussion

Genetic erosion in *Populus nigra* populations in the Czech Republic is considered to be caused by *P.* x *canadensis* (MOTTL, 1989). The level of introgression can be estimated on the basis of hybrid frequency. Three enzyme systems were chosen for the identification of these hybrids. These systems allow an easy differentiation of the mentioned hybrids (RAJORA, 1986; POSPÍŠKOVÁ et al., 1997; JANSSEN, 1997). Considering the hybrid origin from the test crossing *P.* x *canadensis* x *P. nigra* the possibility of segregation ratio 1:1 in the progeny, is probable. This segregation was shown for two enzyme systems, 6-PGD and LAP. A segregation ratio of 3:1 appeared in the GPI enzyme system in favour of the homozygous phenotype *P. nigra.* Both species *P. nigra* and *P.* x *canadensis* are most probably diploid (FEDOROV, 1969). It is probably a case of an abnormal segregation, observable in many isozyme loci (WEEDEN and WENDEL, 1989). RAJORA (1990) found in other loci of the genus *Populus* a discrepancy in observed segregation ratios compared with the expected.

Another problem is the higher number of bands in locus Gpi-2 (*Figure 1*). It was stated by GOTTLIEB (1982) that the enzyme GPI is a dimer. According to TORRES et al. (1986) it is possible that this locus is under control of two gene loci – presumed gene duplication. This could explain the appearence of the higher number of bands in this locus.

It was possible to estimate the probability of hybrid detections on the basis of the obtained segregation ratios and demonstrated independent trait combination ability of 6-PGD and LAP. In combinations 6-PGD/GPI and LAP/GPI we could only assume this independence. The increased described by JANSSEN (1997) was confirmed.

An analysis of three natural populations was conducted on the basis of these results. In the population consisting of the progeny of three *P. nigra* female plants introgression was not found, even if male trees of *P.* x *canadensis* were present in the vicinity. This could be due to the fact that the expected introgression originates from pollen transfer of *P. nigra* onto female plants of *P.* x *canadensis* (BISOFFI, pers. comm.; JANSSEN, 1998). This could also be connected with another fact that the hybridisation of *P. deltoides* and *P. nigra* is possible only in the case when *P. deltoides* is the female parent (MELCHIOR and SEITZ, 1968; ZSUFFA, 1975). But a reciprocal pollen transfer can not be excluded, as we can observe in our controlled crossings. The significance of these results may be limited due to the fact that the seedlings originated from only one year and from two localities.

Hybrids distinguishable from *P. nigra* in morphological traits were found in the population of two-years-old seedlings. The observed darker tips of 1-year old shoots were in accordance with the colour of some individuals in the controlled crossing of *P.* x *canadensis* 'Marylandica' x *P. nigra.* The 9.7% introgression in the population of 10 to 18 years old trees was remarkable. The contemporary tree population went through an environmental selection and also through competitive pressure among individuals of the same species during several years. The fact, that the hybrid individuals correspond to *P. nigra* in morphological traits is also remarkable.

According to the obtained results, introgression from *P.* x *canadensis* in young populations of *Populus nigra* of unknown origin can amount up to 10%. In progenies, derived from identified female trees of *P. nigra,* introgression was absent or was not detectable.

If future experiments confirm these results, this would imply that female plants of *P. nigra* can re-establish populations from seeds, without any important danger of genetic erosion of the species. Thus given the probabilities obtained in the studied population, no introgression from *P.* x *canadensis* was found.

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Spatial Genetic Structure of Allozyme Polymorphisms Within Populations of *Rhus trichocarpa* (Anacardiaceae)

By M. G. CHUNG[1]), J. M. CHUNG[1]) and B. K. EPPERSON[2])

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Abstract

Rhus trichocarpa MIQ. (Anacardiaceae) is a dioecious, insect-pollinated tree. It has a wide distribution in East Asia, from Japan, China and Korea, north to Sakhalin. In these regions, *R. trichocarpa* is a pioneer tree that, once established, creates conditions favorable for subsequent successional tree species, by providing attractive perching for various bird species, which then drop seed of the later successional species. Later species form pine-oak forests which overgrow *R. trichocarpa.* We used allozyme loci and spatial autocorrelation statistics to examine the spatial distribution of allozyme polymorphisms of individuals in two Korean populations. Populations of the species maintain moderate levels of allozyme variation (mean H_e = 0.173, G_{ST} = 0.064). It was found that genetic patch width was at least 25 m, and this was created by limits to seed or pollen dispersal.

Key words: Allozymes, *Rhus trichocarpa,* spatial autocorrelation, spatial genetic structure.

Introduction

Rhus trichocarpa MIQ. (Anacardiaceae) is a dioecious, insect-pollinated woody species. Flowers are visited by bees (*Bombus diversus diversus* and *Apis mellifera,* M. G. CHUNG pers. obs.). The species occurs widely in East Asia, from Japan, China and

[1]) Department of Biology, Gyeongsang National University, Chinju 660–701, Republic of Korea

[2]) Department of Forestry, Michigan State University, East Lansing, MI 48824, USA