Identification of F_1 Hybrids *Pinus nigra* J. F. Arnold × *P. sylvestris* L., *P. nigra* J. F. Arnold × *P. densiflora* Siebold et Zucc. and *P. nigra* J. F. Arnold × *P. thunbergiana* Franco by Internal and External Morphometric Traits

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Summary

Nineteen needle and shoot morphological and anatomical traits of three F_1 hybrids (*Pinus nigra* J. F. Arnold × *P. sylvestris* L., *P. nigra* J. F. Arnold × *P. densiflora* Siebold et Zucc. and *P. nigra* J. F. Arnold × *P. thunbergiana* Franco), and their parental species were analysed using discriminant analyses to distinguish parental species and hybrids.

It was possible to differentiate between the F_1 hybrid *P. nigra* × *P. sylvestris*, *P. nigra* × *P. densiflora* and *P. nigra* × *P. thunbergiana* with complete certainty. Only the hybrids *P. nigra* × *P. sylvestris* can be misidentified with the hybrids *P. nigra* × *P. densiflora* in 3 percent of cases. The hybrids can with 95–100 percent certainty be differentiated from their parental species, except for the hybrid *P. nigra* × *P. thunbergiana* whose probability of misidentification with the European black pine is 23 percent.

For the hybrids it was established which of the analysed traits contribute the most to their differentiation from other hybrids and their parental species; the combination of these traits is specific for each hybrid and cannot be used for other hybrid combinations.

Key words: Pinus nigra J. F. Arnold, *P. sylvestris* L., *P. densiflora* Siebold et Zucc., *P. thunbergiana* Franco, F_1 hybrids, morphological traits, anatomical traits, discriminant analysis, differentiation, identification.

Introduction

Interspecific hybridization in forest trees has been an ongoing interest to forest geneticists and tree improvement scientists studying the mechanics of incompatibility and the feasibility of transferring traits among species (DUFFIELD, 1952). Over three decades (1958–1991) of controlled pollination experiments at the Department of Forest Genetics and Dendrology (Faculty of Forestry, University of Zagreb) has produced a number of putative hybrids between European black pine (*Pinus nigra* J. F. Arnold), Scotch pine (*P. sylvestris* L.), Japanese red pine (*P. densiflora* Siebold et Zucc.) and Japanese black pine (*P. thunbergiana* Franco). Various hybrid combinations were produced, e.g. F_1 and F_2 hybrids, trispecies hybrids and backcross generations (VIDAKOVIĆ and ASSOCIATES, 1973, 1977, 1985, 1991).

Over the years, fourteen experimental plantations of different putative hybrid combinations with parental controls were established in Croatia. Studies have been conducted in these plantations on growth (VIDAKOVIĆ, 1966; VIDAKOVIĆ and BORZAN, 1991; BORZAN *et al.,* 1995; IDŽOJTIĆ, 1996), incompatibility (ĐURBADIĆ *et al.,* 1967, 1973, 1977; PETRIČEVIĆ *et al.,* 1977; VIDAKOVIĆ, 1963; VIDAKOVIĆ and BORZAN, 1973; VIDAKOVIĆ, 1977a, b; VIDAKOVIĆ and JURKOVIĆ-BEVILACQUA, 1970), and cytogenetics (VIDAKOVIĆ, 1958; BORZAN, 1981, 1984, 1987, 1988). Different morphometrical research and analysis of essential oils from the needles were carried out (BORZAN and IDŽOJTIĆ, 1996; IDŽOJTIĆ, 1996, 1997; IDŽOJTIĆ and PFEIFHOFER, 2001).

In case of so many different produced hybrids and hybrid combinations in established 14 experimental trails, identification and confirmation of hybridity, however, has been difficult due to similarities in habit, growth and morphological traits among hybrids and their parental species. First attempt to use discriminant analysis in differentiating pine hybrid families in experimental plots in Croatia was carried out by IDŽOJTIĆ (1996). Based on three analysed traits of the needles (needle length, number of needle serrations along one margin, number of dorsal stomatal rows) it was impossible to differentiate the F_1 hybrids *nisy* both from their parental species and from the F_2 hybrids *nisy*. BORZAN and IDŽOJTIĆ (1996) increased the number of variables in the discriminant analysis from three to five (the features DBH and tree height were added) that increased the accuracy of F_1 hybrid identification from 53 percent (IDŽOJTIĆ, 1996) to 66 percent, which still was not significant.

These previous studies revealed that to ascertain hybrids from their parental species it was necessary to employ a combination of diagnostic traits. In this study we compared F_1 hybrids *P. nigra* × *P. densiflora* and *P. nigra* × *P. thunbergiana* with parental species and with F_1 hybrids *P. nigra* × *P. sylvestris* by chosing nineteen morphological and anatomical traits of needles and shoots for discriminant analyses. The purpose was to distinguish many of those hybrid combinations from their parental species and to establish which of the analysed traits contribute the most to the identification of certain hybrid combination and which combination of traits is specific for each hybrid.

Material and Methods

Branch samples were taken from four different pine species (*P. nigra* = *ni*, *P. sylvestris* = *sy*, *P. densiflora* = *de*, *P. thunbergiana* = *th*) and three F_1 hybrids of these species (*P. nigra* × *P. sylvestris* = *nisy*, *P. nigra* × *P. densiflora* = *nide*, *P. nigra* × *P. thunbergiana* = *nith*). The sampled trees were located in the experimental plots in Đurđevački peski (four plots), in Lisičine Arboretum (five plots), and plots at the University of Zagreb. Two shoots from each tree, with completely developed one-year needles, were picked in end of October 1996. For each type (species or hybrid) samples were taken from as many different trees as possible, however, the number of samples per type varied according to plot size and survival at the different locations. For the F_1 hybrids: *nisy* samples were taken from 32 trees, 29 trees for *nide*, and 15 trees for *nith*. For the analysis of *ni* branches were picked from 41 trees, for *sy* from 29 trees, for *de* from 40 trees, and for *th* from 9 trees.

To differentiate among hybrids and pure species, nineteen different traits from the needle and shoot samples were analy-

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sed: 1. needle length = *NL* (cm); 2. fascicle sheath length = *FSL* (cm); 3. tracheid length = *TL* (mm); 4. tracheid width, in the middle of tracheid, from one year old shoot = *TW* (μm). The shoots were macerated by boiling 1-2 minutes in 10 % HNO_3 (GERLACH, 1969), tracheids isolated and fixed on slides; 5. number of ventral stomatal rows = *NVSR;* 6. number of dorsal stomatal rows = *NDSR;* 7. number of stomata per 1 cm of one ventral stomata row = *NS/cm*. Each needle-cut was 1 cm long, 0.5 cm above and below the middle of the needle. The mid-row of the needle was used if possible, unless the stomatal row was broken. In that case, the closest, non-interrupted stomatal row was used. 8. number of needle serrations per cm along one margin in the middle of the needle = *NNS/cm;* 9. needle cross-section area in at the mid-length of the needle = *NA* (mm^2). Each cross-section was used to measure traits described under numbers 9–14. Permanent samples of the needle cross-sections were made. 10. needle cross-section height = *NH* (mm); 11. needle cross-section diameter = *ND* (mm); 12. stelar region cross-section area = *SRA* (mm^2); 13. stelar region cross-section height = *SRH* (mm); 14. stelar region cross-section diameter = *SRD* (mm); 15. maximum number of hypodermal cells layers = *NHLmax.* Since the number of hypodermal layers varies, only the maximum number of layers in each cross-section was recorded; 16. number of medial resin canals = *NMRC;* The resin canals in *sy* and in *de* are placed along the hypodermis, and only some are placed medially. For these species as the measure of central tendency for the *NMRC* trait, the mode and median were calculated, and as the measure of variability the interquartile range was calculated (range $Q_3 - Q_1$). 17. number of external resin canals = *NERC.* In *ni, th* and *nith*, the resin canals are placed medially, and only some are placed along the hypodermis. Thus, the mode, median and interquartile range were calculated. 18. maximum number of sheath cells surrounding a single resin canal in each cross-section = *NSCmax;* 19. minimum number of sheath cells surrounding a single resin canal = *NSCmin.*

Light microscopic investigations were done with an Axioskop microscope II (Zeiss Inc.) equipped with a DXC 950 P videocamera (Sony Inc.), on a PCI framegrabber (Image Technology Inc.), using Media Cybernetics Inc. image analysis software. Stomatal rows were counted under a binocular magnifying lens. Data were processed by discriminant analysis using the statistical package StatSoft. Inc (2001).

Three separate discriminant analysis, using all 19 traits were conducted. Each F_1 hybrid was compared with the parental species and the other F_1 hybrids. The independent variables were entered into the model by the forward stepwise method. The tolerance value was 0.01. A fourth discriminant analysis was conducted to show the relationship of all analysed hybrids and parental species.

Results

Descriptive statistics

Inspection of *Table 1* shows some hybrid traits are intermediate, while others are not. For instance, needle length (*NL*) of the hybrid *nisy* shows intermediacy when compared to the needle length of parental species. However, needles of *nide* hybrids are longest; significantly longer from *P. densiflora*, but nonsignificantly longer from *P. nigra* (shown after detailed statistical analysis, which is not presented in this paper due to over sizing the paper length). On the contrary, hybrid *nith* has significantly shorter needles than both parental species.

First Discriminant Analysis (nisy, ni, sy, nide and nith)

Discrimination among the groups was found to be significant (Wilks' $\lambda = 0.0172$; $F\ (76, 1058) = 25.1$; $p < 0.01$). All the nineteen independent variables were included into the model. Based on the nineteen analysed traits the F_1 hybrid samples *nisy* were classified as *nisy* hybrids in 92.2 percent (*Table 2*). They were completely differentiated from *sy* and from the hybrid *nith*. The probability of misidentification of the hybrid samples *nisy* with *ni* is 4.7 percent, and with the hybrids *nide* 3.1 percent.

Second Discriminant Analysis (nide, ni, de, nisy and nith)

Discrimination among the groups was again found to be significant (Wilks' $\lambda = 0.0142$; $F\ (76, 1144) = 29.3$; $p < 0.01$). *Table 2* shows that the F_1 hybrid samples *nide* were classified as *nide* hybrids in 94.8 percent. They were misidentified with

Table 1. – Statistical parameters of nineteen analysed morphological and anatomical traits for F_1 hybrids *nisy, nide, nith* and parental species *ni, sy, de* and th. $\bar{x}$ = arithmetic mean, *CV* = coefficient of variability (%), *N* = sample size.

Trait	*nisy* N = 64		*nide* N = 58		*nith* N = 30		*ni* N = 82		*sy* N = 58		*de* N = 80		*th* N = 36	
	$\bar{x}$	*CV*	$\bar{x}$	*CV*	$\bar{x}$	*CV*	$\bar{x}$	*CV*	$\bar{x}$	*CV*	$\bar{x}$	*CV*	$\bar{x}$	*CV*
NL (cm)	10.9	22.3	13.0	18.4	10.2	19.0	12.4	26.2	9.6	15.3	12.0	15.5	12.7	21.0
FSL (cm)	0.9	22.5	1.1	22.3	1.0	14.7	1.0	20.0	0.8	13.3	1.0	17.3	1.1	14.6
NVSR	10	20.4	7	17. 6	7	17.4	8	15.9	13	21.5	7	16.8	7	14.7
NDSR	14	21.6	11	19.6	12	14.8	12	16.7	15	18.7	10	18.6	13	12.8
NS/cm	106	10.1	104	11.4	99	6.8	101	9.2	115	8.7	119	8.0	92	9.2
NNS/cm	29	23.0	35	22.7	37	23.8	32	20.8	37	20.3	54	14.6	60	13.0
NA (mm^2)	1.0490	19.1	0.8180	19.4	0.9799	18.5	1.0031	19.8	0.9956	22.1	0.5935	15.2	1.0056	20.0
NH (mm)	0.918	12.8	0.873	12.6	0.998	11.9	0.959	13.3	0.818	12.9	0.733	11.0	1.058	14.0
ND (mm)	1.573	10.8	1.313	9.9	1.414	10.5	1.445	10.3	1.647	13.0	1.135	7.8	1.379	10.0
SRA (mm^2)	0.3057	19.3	0.2153	23.2	0.2501	17.7	0.2724	21.2	0.3083	24.1	0.1479	22.7	0.2501	23.5
SRH (mm)	0.417	10.0	0.376	10.6	0.424	10.4	0.427	10.7	0.371	11.9	0.319	13.2	0.452	13.3
SRD (mm)	0.931	13.0	0.717	14.1	0.745	11.6	0.796	12.7	1.041	14.9	0.591	10.6	0.704	12.7
NHLmax	2.3	21.6	2.4	20.6	3.1	23.3	3.4	20.6	1.3	35.7	1.3	35.6	3.1	18.0
NMRC	6.0	56.6	5.6	39.8	5.5	31.3	6.0	45.0					4.6	45.6
NERC	4.5	71.4	2.1	93.1					11.5	20.5	6.5	22.3		
NSCmax	14.1	22.2	13.9	15.2	12.8	17.8	13.4	15.9	18.7	25.2	11.6	18.6	11.3	17.3
NSCmin	7.7	17.9	8.5	14.0	7.9	11.1	8.3	16.7	7.7	26.3	7.3	23.8	7.7	18.7
TL (mm)	1.074	14.2	1.065	16.3	1.219	18.1	1.055	10.8	1.030	10.8	1.232	12.5	1.430	10.7
TW (μm)	23.1	13.0	21.1	9.7	26.9	19.5	24.1	12.5	25.1	14.3	21.9	12.0	26.5	9.8

Table 2. – Classification matrix. According to classification functions, all measured data were classified into groups (by rows in the table) to which they are most likely to belong. No. = number of samples.

F_1 Hybrid	Correct Classified as		Misclassified as								Sample Size
	No.	%	No.	%	No.	%	No.	%	No.	%	
	nisy		*ni*		*sy*		*nide*		*nith*		
nisy	59	92.2	3	4.7	0	0	2	3.1	0	0	64
	nide		*ni*		*de*		*nisy*		*nith*		
nide	55	94.8	2	3.4	1	1.8	0	0	0	0	58
	nith		*ni*		*th*		*nide*		*nisy*		
nith	22	73.4	7	23.3	1	3.3	0	0	0	0	30

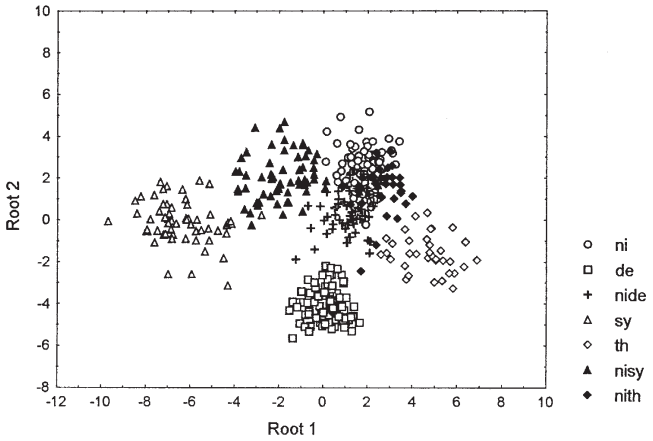

Figure 1. – Scatterplot of the canonical scores. Particular values for the first discriminant function are plotted on axis x (root 1), and for the second discriminant function on axis y (root 2).

ni in 3.4 percent of cases, and with *de* in 1.8 percent of cases. Based on combination of nineteen traits, the samples of the hybrid *nide* were completely differentiated from the other two hybrid combinations, *nisy* and *nith*.

Third Discriminant Analysis (nith, ni, th, nisy and nide)

As with the previous analyses, discrimination among the groups was found to be significant (Wilks' $\lambda = 0.0177$; F (76, 971) = 22.8; $p < 0.01$). Samples of the F_1 hybrid *nith* were classified as *nith* in 73.4 percent (*Table 2*). They were misidentified with the samples of *ni* in 23.3 percent of cases. Also the misidentification of the hybrid *nith* with *de* is possible with a probability of 3.3 percent. The other two hybrids, *nide* and *nisy*, were completely differentiated from the samples of the *nith* hybrid.

The discriminant analyses showed that all three F_1 hybrids could be misidentified with the female parent *ni* in most often cases as: the *nith* hybrid in 23.3 percent of cases, *nisy* in 4.7 percent, and the *nide* hybrid in 3.4 percent of cases, respectively. Additionally *nisy* hybrids were misidentified as *nide* hybrids (3.1 percent), *nide* hybrids as *de* (1.8 percent), and *nith* hybrids as *th* (3.3 percent), i.e. in all cases except for the *nith* hybrid parental species and hybrid combinations were sufficiently distinct to avoid misidentification.

Fourth Discriminant Analysis

Combining data from previous three analyses into the fourth discriminant analysis for all hybrids and parental species together, graphical presentation of the results clearly shows grouping of parental species and placing of hybrids between each respective parental group (*Figure 1*). Statistical parameters were: Wilks' $\lambda = 0.0024$; F (114, 2206) = 35.6; $p < 0.01$.

As a result of discriminant analyses of nineteen analysed morphological and anatomical traits of needles and shoots in investigated hybrids and parental species, *Table 3* revealed that five traits (out of 19) can be used to differentiate hybrids from each other and from their parental species in these groups. The most important traits for the differentiation are: size of needle cross-section (*NA* and *ND*), cross-section of the central cylinder (*SRA* and *SRD*) in the middle of needle length, and number of medial resin canals (*NMRC*) respectively, as seen from the trait 1 in *Table 3*.

Table 3. – Five traits that F_1 hybrids most easily differentiate from their parental species and from each other.

Groups	1. Trait	2. Trait	3. Trait	4. Trait	5. Trait
nisy - nide	*NA*	*SRA*	*SRD*	*FSL*	*NMRC*
nisy - nith	*SRD*	*SRA*	*NMRC*	*NNS/cm*	*ND*
nide - nith	*ND*	*NA*	*SRD*	*NH*	*NMRC*
nisy - ni	*NA*	*SRD*	*NERC*	*NMRC*	*NHLmax*
nisy - sy	*SRA*	*NA*	*SRH*	*NSCmax*	*NNS/cm*
nide - ni	*NMRC*	*SRA*	*SRH*	*NHLmax*	*TW*
nide - de	*NA*	*NNS/cm*	*ND*	*NMRC*	*TL*
nith - ni	*ND*	*NA*	*NH*	*SRD*	*NL*
nith - th	*SRA*	*NNS/cm*	*TL*	*SRH*	*SRD*

Discussion

MERGEN and FURNIVAL (1960) have used stepwise discriminant analysis to differentiate hybrid seedlings of *P. thunbergiana* × *P. densiflora* from their parental species, using 27 characters. They considered the method likely to be of great value in forest biology research and desirable in obtaining information on the overall appearance and development of the hybrid plants. Our first experiences in differentiating pine hybrids were with small number of analysed variables (IDŽOJTIĆ, 1996; BORZAN and IDŽOJTIĆ, 1996), but this study has shown that with larger number of analysed traits the results were more significant. In this study we were able to distinguish significantly three analysed hybrids and to differentiate them from their parental species, with the exception of the hybrid *nith*, which was not possible to differentiate from the *ni* parent in 23.3 percent of cases (*Table 2*). It is obvious that in this case additional or different traits will be needed to distinguish this hybrid from parental species with accuracy. KRIEBEL (1962) stated that each pine hybrid has a different trait combination, which makes it most easily distinguishable, i.e., that a once established combination of traits for one interspecial hybrid cannot be applied for hybrids of other pine species. His opinion is confirmed by our study as shown in *Table 3*. It is clear also that the only one trait considered as the best one for identification is not enough. Each other trait used for identification is less accurate, but contributing in final identification with certain percentage.

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Identification of *Pinus elliottii* var. *elliottii* X *P. caribaea* var. *hondurensis* Hybrids Using the Chloroplast *trnL-F* intergenic Spacer

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Summary

Breeding programs based on hybrids of tree species often have a need to unambiguously distinguish intraspecific and self progeny from hybrid progeny. The interspecific hybrid of *P. elliottii* var. *elliottii* (PEE) and *P. caribaea* var. *hondurensis* (PCH) is difficult to reliably distinguish from pure PEE based on morphology, especially at a young age. We examined the *trnL-F* intergenic spacer region of the paternally inherited chloroplast genome for a polymorphism that may distinguish these two taxa. Sequencing this region indicated there were two haplotypes, one which was specific to PEE, designated (+), the other designated (–), was shared by both taxa. This result was consistent with other studies which suggest past introgression of PCH into PEE. A PCR assay was developed to detect the PEE specific haplotype. This haplotype was found at a frequency of 0.6 in a sample of 22 PEE from a breeding population but was absent from a sample of 30 PCH parents. As expected, hybrids from crosses of PCH pollen donors and maternal (+) haplotype PEE had (–) haplotypes. In situations where the mother can be genotyped, and the pollen pool consists of maternal (self) or PCH pollen, this assay would unambiguously determine the hybrid status of offspring from 60 % of the PEE

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