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Putative hybridization between P. caribaea Morelet and P. oocarpa Schiede: a canonical approach[1])

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Summary

Principal component and canonical correlation analyses were applied to three sets of traits (needle, cone and chemical compounds) measured on a sample of pine trees. A transect in a location at Honduras was defined were *P. caribaea* MORELET and *P. oocarpa* SCHIEDE live sympatrically. Results suggest that natural hybridization is occurring between these species. Intermediate and outlying trees were statistically found. Intermediate trees have characteristics between the two parent populations and outliers are those sharing characteristics of both parental populations but for different sets of traits.

Key words: Hybridization, *P. caribaea, P. oocarpa*, principal components, canonical correlations.

Zusammenfassung

Die Hauptkomponenten-Analyse und die kanonische Korrelationsanalyse wurden auf drei Merkmalsgruppen (Kiefernnadeln, Kiefernzapfen und chemische Zusammensetzung des Holzmaterials) angewandt, die anhand einer Stichprobe von Kiefern gemessen wurden. In Honduras wurde ein Transect festgelegt, wo *P. caribaea* MORELET und *Pinus oocarpa* SCHIEDE nebeneinander vorkommen. Die Ergebnisse lassen den Schluß zu, daß eine natürliche Hybridisierung zwischen beiden Arten vorkommt. Intermediäre und Ausreißerformen konnten statistisch abgesichert unterschieden werden. Die intermediären Formen weisen Merkmale auf, die in ihrer Ausprägung zwischen denen der beiden Elternpopulationen liegen, während die Ausreißerformen sich Merkmale beider Elternpopulationen teilen, jedoch für verschiedene Merkmalsgruppen.

1. Introduction

These two pine species are industrially important because they are fast growing trees and in short periods of about 15 to 20 years they yield economic quantities of wood, resin and seed. In some countries these species can be used in fuelwood plantations for energy (BURLEY, 1980). Total natural areas approximate to 2.5 million ha. and the plantation area exceeds 1 million ha. in 57 countries.

It has been suggested that *P. caribaea* MORELET and *P. oocarpa* SCHIEDE may cross producing fertile offspring (WILLIAMS, 1955; MIROV, 1967). This motivated research on the possible hybridization of the two species developed at the Commonwealth Forestry Institute (CFI), Oxford, (UK) and the Tropical Products Institute (TPI), London.

A natural stand of *P. caribaea* var. *hondurensis* and *P. oocarpa* in Pinalejo is being studied. This paper is a follow up of the results published by STYLES (1976); FERNANDEZ DE LA REGUERA *et al.* (1984) and STYLES *et al.* (1982), where descriptions of the sampling procedures, the individual trees and their environment are presented. Statistical multivariate methods used in these references are mainly, principal component and canonical correlation analyses applied to selections of needle and cone measurements. This paper introduces the chemical compounds evaluated per tree and a different selection of variables based on a statistical screening process.

2. Material and Methods

The data were obtained from trees selected along a transect in Pinalejo, Honduras (15°23'N, 88°24'W). Altitude along the transect ranged from 300 to 900 m (approx.) above sea level. Ten altitude ranges, which were considered as sites, were defined, their limits not exactly fixed because of the natural spread of the trees and their availability at specific altitude. Ten trees were randomly selected per site, from those that were apparently healthy, mature and dominant, and aged 20 years or more.

The actual data is an extension of that used in Styles' papers. It includes the two bottom sites which were not considered by STYLES, because he thought they were stands of pure *P. caribaea* trees, and a determination of the chemical compounds in the sampled trees.

A large number of variables were evaluated but a statistical screening of them reduced their number to 22 namely needle width (NWD), number of needles per fascicle (NNDL), length of sheath (SHEZ), number of stomatal rows on the dorsal side (STD), ventral side (STV) and in 5 mm section of the needle (ST5MM), total number of resin canals (RES); cone length (CLNG), cone diameter (DIAM), width at mid point of cone (WMD), distance to widest point of cone (DSWP), width of the umbo (UMBO), shape of cone (SHAPE); α-pinene (APAIN), myrcene (MYRC), limonene (LIMO), β-phellandrene (BFEL), allo-occinene (ALLO), camphor (CAM), longifolene (LFOL) and longicyclene (LCYC). These variables are those showing a linear trend

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of the site means along the transect and their definitions can be found in previous reports on this material (STYLES *et al.*, 1982; FERNANDEZ DE LA REGUERA *et al.*, 1984).

The number of resin canals variable corresponds to the total number of resin canals, regardless of their position in the central portion of the needle and shape of cone was defined as cone length divided by diameter. This definition of cone shape although strongly correlated to that proposed by STYLES et al. (1982) has the advantage of being a scale-free index.

Morphological measurements were done at the CFI, Oxford, Chemical compounds are expressed as percentage of peak in distilled oil produced by GLC and recordings were obtained at TPI, London, with a HP 7620 A apparatus with injection at 75° C and increased 4° C per minute to 220° C and then held constant.

A prior classification of the trees into *P. caribaea* and *P. oocarpa* species and uncertain was done by Dr. B. T. STYLES, a professional pine taxonomist. This morphological classification was not considered in the analyses but used for comparison.

Statistical multivariate methods applied were principal component analysis (PCA) and the standard form of canonical correlations analysis (CCA) first developed by HOTELLING (1936). Both techniques are well described alsewhere (see e.g. MARDIA *et al.*, 1979).

3. Results

3.1 *Principal component analyses*

PCA analyses were done on the correlation matrix of several sets of the variables, the vector elements corresponding to the correlation between the raw variables and the respective PC. Sets consisted of needle, cone and chemical variables.

Results showed that the sample could be divided into groups by the first PC only. *Table 1* presents the essential PCA results; circles in this table denoting relatively high coefficients.

Reification of the principal component in each analysis was: number of stomatal rows in needles; shape of cone; α-pinene; respectively for needle, cone and chemical traits sets.

Scatter plots of the first two PC and the histogram corresponding to the first PC, for each of the three analyses, are presented in *Figure 1*. Trees are depicted according to the prior classification.

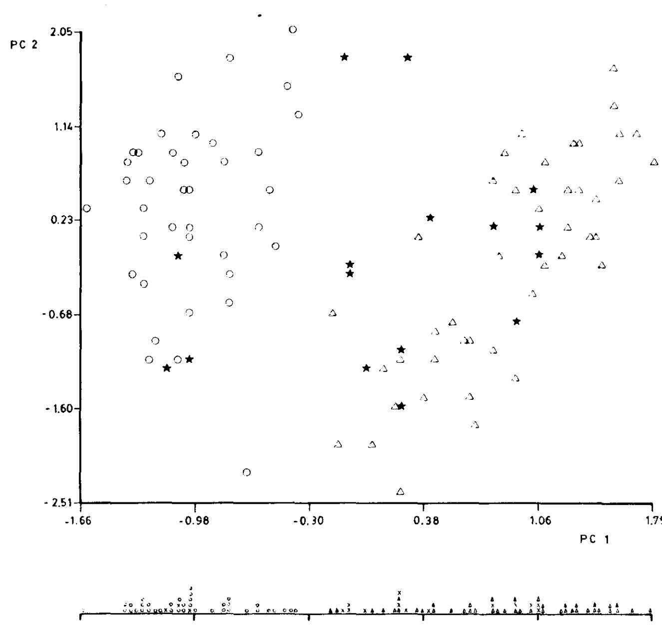
Table 1. — Roots explained variation and coefficients in PCA on needle, cone and chemical sets.

	Needles		Cones		Chemicals
ROOT	3.770		2.735		5.092
% of TR	47.13		45.58		63.65
NLNG	.548	CLNG	.985	APIN	.289
NWD	.891	DIAM	.178	MYRC	-.758
NNDL	-.846	WMD	.480	LIMO	-.861
SHEZ	-.282	SDWP	.895	BFEL	-.740
STD	.954	UMBO	.106	ALLO	.875
STV	.739	SHAPE	.831	CAM	.899
ST5MM	-.468			LFOL	.910
RESN	-.452			LCYC	.863

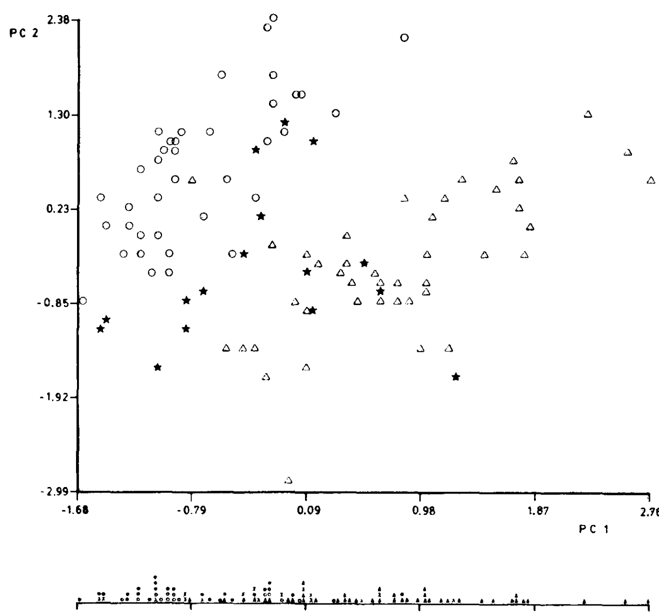
It is not difficult to distinguish groups of observations which are defined according to the first PC, on needle variables. Groups of observations are not clearly distinguishable when cone or chemical variables are considered on their own. If we rely on the prior classification, *Fig. 1* (c) suggests a high correlation between α-pinene and the remaining chemical for *P. oocarpa* trees.

Grouped observations can be seen to correspond, at least roughly, with *P. caribaea* and *P. oocarpa* species according to BTS classification.

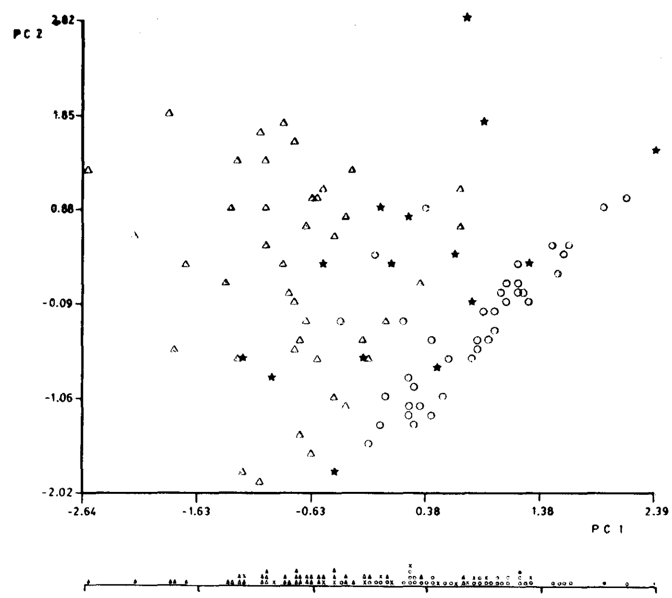
The important result from PCA was the presence of some intermediate trees. Boundaries for *P. caribaea* and *P. oocarpa* trees could be assessed. This produced a classification of the sampled trees into those belonging to each species and intermediate ones not allocable to either species. These intermediate trees could be statistically determined hybrids but, the relatively small percentage of variation



a) PCA on needle set



b) PCA on cone set



c) PCA on chemical set

Prior classification: △ *P. caribaea* ○ *P. oocarpa* * uncertain

Figure 1. — Principal components analysis. Scatter plots for first pair of PC vectors and histograms for first PC.

explained by the first principal components made this statement dubious.

3.2 Canonical correlation analyses

In all these analyses, only the first CC was significant according to MARRIOTT's (1952) test. The second CC was always far from the usual 0.05 significance test level. CCA were done on the respective correlation matrix. A subjective form of assessing the relative weight of a particular CC is to calculate its proportion of the sum of all the canonical roots, which is the trace of the matrix of canonical roots. *Table 2* presents the CCA basic results for the three analyses. The first two roots only are presented for each.

Table 2. — Tests on canonical correlation analysis on all three cases.

CCA	Root	CC	% of tr	MARRIOTT'S TEST CHI-SQ	DF	PROB
Needle vs	.624	.790	61.9	90.85	18.35	0.00
cone	.183	.427	18.1	18.66	15.16	0.20
Needle vs	.767	.876	59.8	133.93	21.70	0.00
chemical	.164	.405	12.8	16.60	18.45	0.60
Chemical	.690	.831	64.1	108.65	18.35	0.00
vs cone	.183	.428	17.0	18.75	15.16	0.20

Reification of the canonical vectors was done considering relatively large coefficients in the canonical vectors, as for PCA. Canonical vectors are presented in *Table 3* — circles marking relatively large large values.

Canonical vectors corresponded then to: a) CCA on needle *vs* cone sets, CV1 is STD; CV2 is DDWD and DIAM; b) CCA on needle *vs* chemical sets, CV1 is SHEZ and STD; CV2 is LIMO, ALLO and LFOL; and c) CCA on chemical *vs* cone sets, CV1 is LIMO, ALLO and LFOL; CV2 is shape of cone.

Correlations among chemical variables were relatively large, particularly between MYRC and BFEL (r = 0.98) and their presence in the canonical vectors with similar coefficients of different signs suggested that the chemical

Table 3. — First pairs of canonical vectors for all three CC analyses.

A) CCA Needle vs. cone sets

CV1:	NLNG	NWD	NNDL	SHEZ	STD	STV	ST5MM	RESN
	.1238	.0441	-.1945	-.2671	.6110	-.0492	.0022	-.1219
CV2:	CLNG	DIAM	WMD	DSWD	UMBO	SHAPE		
	-.2120	-.5223	.3559	.6339	.0179	.3697		

B) CCA Needle vs. chemical sets.

CV1:	NLNG	NWD	NNDL	SHEZ	STD	STU	ST5MM	RESN
	-.0817	-.1391	.1640	.3750	-.4422	.0425	.0653	.1742
CV2:	APIN	MYRC	LIMO	BFEL	ALLO	CAM	LFOL	LCYC
	.0624	-1.2838	-.7326	1.1527	-.6131	.0445	.8918	-.2753

C) CCA Chemical vs. cone sets

CV1:	APIN	MYRC	LIMO	BFEL	ALLO	CAM	LFOL	LCYC
	-.2330	1.7880	.9638	-1.9453	.6618	-.2971	-.4189	.1172
CV2:	CLNG	DIAM	WMD	DSWD	UMBO	SHAPE		
	-.5265	-.5682	.5531	.6623	.0841	.5817		

canonical vectors were loaded on limonene, allo-ocimene and longifolene contents.

Plotting of observation scores for pairs of CV is interesting for they give a picture of the sample in a bi-dimensional space. The scatter plots for the three CCA are presented in *Figure 2*. Each CV is a one-dimensional representation of the sample in the respective variable space and the two canonical vectors are linked by the canonical correlation, which is the cosine of the angle formed by them. Their scatter plots then, are different from those produced with PC scores. In PCA the coordinate axes are orthogonal.

The scatter plots (*Fig. 2*) did produce groupings of the observations with a number of trees in intermediate position and some showing themselves as outliers. Intermediate trees are those plotted along the 45° trend linking the groups of possible *P. caribaea* and *P. oocarpa* trees while outliers are those trees away from the trend and sharing the characteristics of one species for a set of variables (e.g. they are *P. caribaea* type for needles) and the characteristics of the other species for the other set of variables (e.g. they are *P. oocarpa* type for cones) in the same CCA.

4. Conclusions

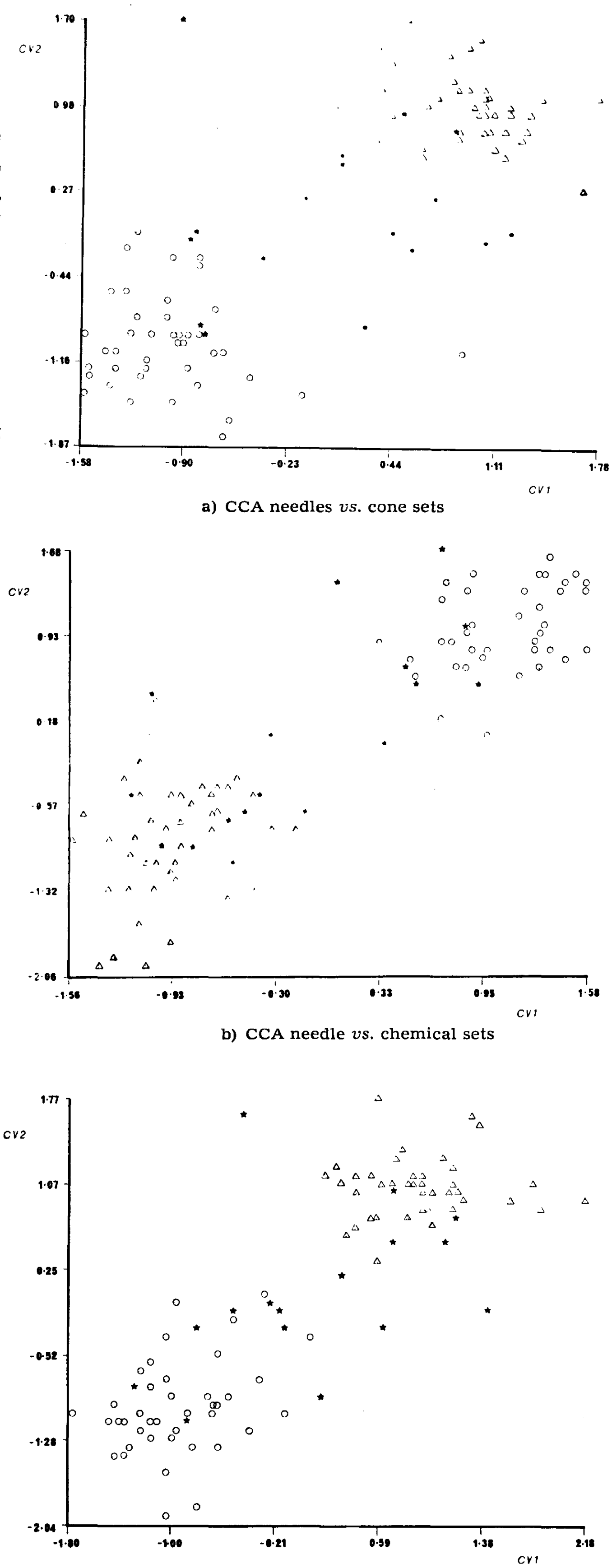
The definition of resin canals as their total number, regardless of their position in the central portion of the needles, has not been widely used in statistical taxonomy and this variable had no significant weight in the combination of variables produced by the statistical methods applied, so that its inclusion in further research is not justified.

The selection of chemical variables included mainly minor components. Major components in this set of variables, i.e. those producing yields over 10%, were α-pinene and β-phellandrene, chemical components were useful as species indices if considered jointly with needle or cone traits but not on their own. The analyses led to the conclusion that trees of the parent species can be distinguished on the basis of their limonene, allo-ocimene and longifolene contents.

Results from principal component analyses produced a classification of the trees consisting of *P. caribaea*, *P. oocarpa* and dubious trees. It can also be deduced from these analyses that needle measurements can distinguish the two *Pinus species* although they overlap at medium transec altitudes. Cone and chemical measurements individually cannot be used as PC indices for identifying species but, considered jointly with one of the other sets of variables, they proved to be useful.

PC analysis showed two groups in the sample, plus some trees which were classified as intermediate. These analyses showed that the first PC only was meaningful for grouping the observations, and because of this, trees could generally be classified as intermediate or belonging to one of the groups. Outlying observations had to be considered with care for the second PC was not reliable for grouping of observations.

On the other hand, CC plots relate two highly correlated variates. The regression line of one or the other would have a positive slope and would almost coincide with the main diagonal of the plot, so that outlying observations will fall far away from the general trend of the swarm of points. This makes CCA more reliable for detecting hybrids than the PCA provided, of course, that they exist.



a) CCA needles *vs.* cone sets

b) CCA needle *vs.* chemical sets

c) CCA chemical *vs.* cone sets

Prior classification: △ *P. caribaea* ○ *P. oocarpa* * uncertain

Figure 2. — Canonical correlation analysis. Scatter plots for first pairs of canonical vectors.

Table 4. — Classification of sampled trees according to several criteria (dubious cases only).

Transect Altitude	Tree No.	CLASSIFICATION Styles	PCA	CCA	Final
315	1	C	?	C	C
315	5	C	?	C	C
360	20	C	?	C	C
400	25	?C	C	C	C
410	29	?C	C	C	C
475	39	?H	?	C	?C
500	41	?O	O	O	O
505	42	O	O	O	O
505	43	?H	?	C	?C
520	44	?O	?	O	O
530	47	?H	?	?	H
530	50	?H	?	?	H
535	48	?O	O	O	O
535	49	?H	C	C	C
585	55	?O	?	?O	H
585	56	H	C	?H	H
590	51	C	C	C	C
590	53	C	C	C	C
595	57	?C	C	?C	?C
600	58	H	?	?H	H
660	68	?H	?	?O	H
660	69	?H	O	O	O
660	70	C	C	?H	H
710	74	O	O	?O	?O
795	81	?H	?	O	H

General conclusions from our canonical correlation analyses agree with Styles *et al.*, (1982) and Fernandez de la Reguera *et al.*, 1984). Styles *et al.* (op. cit.) classified trees No. 56 as "extreme", but it appears near to the intermediate group, as well as tree No. 58 which is an outlying observation[5]). Both of these were also recorded as outlying cases in the present needle *vs.* cone CCA. Tree No. 56 was also an outlier for needles in the CCA of needles *vs.* chemical sets and tree No. 58 was an outlier in the CCA of chemical *vs.* cone sets.

This clear distinction between trees that are *P. caribaea* type *P. oocarpa* type, intermediate and outliers did not show as clearly in Styles *et al.* paper. In PC and CC analyses when needle or chemical traits are considered, tree No. 58 is suggested to be of *P. oocarpa* type, but if cone traits are considered, it becomes *P. caribaea* type. Analogously, tree No. 70 is *P. caribaea* type for needles and *P. oocarpa* type for cone and chemical variates.

Trees Nos. 50 and 68 sometimes appeared as outlying cases and sometimes intermediate. Observation No. 50 was classified as intermediate or *P. oocarpa* type in CCA while it was classified as *P. caribaea* in PCA. Observation No. 68 can be a *P. caribaea* or a *P. oocarpa* tree according to the CCA considered, but was classified as *P. oocarpa* in PCA.

[5]) The outliers in CCA are trees with *P. caribaea* type needles and *P. oocarpa* cones (or vice-versa).

This situation lead us to state that these trees are more likely intermediate.

The CCA produced a classification of the observations which corresponds to that obtained in PCA, except in some 20 cases, and their results strongly suggest that natural hybridization is occurring at the sampled location.

Table 4 presents the classification of dubious trees according to Styles, PCA and CCA. It also includes a final assessment of the trees. Observations not in the table are *P. caribaea* trees if numbered 50 or less and *P. oocarpa* type if numbered 51 or above.

Standard canonical correlation analyses produced a good classification of the observations in species, intermediate trees and outliers. The presence of outliers in these CC analyses is the strongest support for the hybridisation hypothesis. To detect putative hybrids statistically then, it is necessary to consider the different sets of traits jointly — at this stage, by pairs.

Canonical correlations were high, indicating the strong relationship of dorsal number of stomata rows with shape of cone and with minor chemical compounds, and also between those last two sets of variables. Several outlying and intermediate trees were the same in all the canonical analyses. This consistency ruled out the possibility of these observations being random observations.

The presence of some hybrid trees at relatively high altitude suggests that a possibly new taxon will not be confined to medium altitude ranges but will occupy medium to high altitudes, within the altitudinal range of both species; i.e. from 400 to 800 m.

Since hybrid trees have characteristics associated with both species they are difficult to define. Hybrid trees have, in general, 3 to 5 needles per fascicle; their needle width and number of stomata rows on the dorsal and ventral side are intermediate between the averages of the pure species. The diameter of the hybrid cone is nearly the same as its length and its width at midpoint, but the widest point will be at about the lower third of its longitudinal axis. Hence, the hybrid cone will be "egg-shaped" with an intermediate shape between that of a pure *P. caribaea* tree and that of a typical *P. oocarpa* individual.

The analyses of terpene content were not as conclusive in defining the hybrid type. Hybrid trees contain higher than average yields of minor components like allo-ocimene, longifolene and longicyclene at the expense of the major components α-pinene and β-phellandrene.

It must be pointed out that no statistical analysis of a small number of characters can unequivocally define taxonomic species (which should be based on analyses of the full genetic range of variability in a wide range of characters and requires the skill and experience of professional taxonomits). The intensive sampling and refined statistical methods used in the present study could be expected to identify differences and groupings of individuals that might not appear in standard taxonomic appraisal.

However, most trees at the upper and lower sampling sites were categorically distinct and could be recognised as the previously assessed pure specie while trees at intermediate altitude were intermediate in many characters and in multiple combinations of the traits. These could represent introgressive hybrids between the two species although it is not possible from this small sample to determine the direction of the gene flow. The outliers in the distribution, i.e. trees belonging to different groups depending on the variate considered, are also likely to be F_2 or later generation segregants that maintain some characters of both pure species.

The extent and pattern of hybridisation can be determined fully only after controlled fertilization but the present study provides strong, circumstantial and statistical phenotypic evidence supporting the occurrence of hybridisation between the two taxa that are traditionally considered to be distinct species in different subsections of the genus *Pinus* (*Oocarpeae* and *Australes*).

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Inheritance of Isozyme Variations in Japanese Black Pine, Pinus thunbergii Parl.

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Summary

Inheritance of isozyme variations of 19 enzyme systems in Japanese black pine (*Pinus thunbergii* PARL.) was investigated by polyacrylamide gel electrophoresis. Analysis using haploid megagametophyte tissues was demonstrated that allozyme variants in these enzymes were encoded by a total of 120 structure genes in 37 loci. In five of these loci, *Adh-2*, *Me-2*, *Tzo-3*, *Tzo-4* and *Got-3*, no variation was recognized. In other 32 loci, more than two variants in each loci were found. The confirmation of 1:1 segregation ratio in seed of heterozygous trees revealed that these allozymes exhibited simple Mendelian inheritance. Three sets of isozyme were observed, that were of the same electrophoretic character and were encoded by different loci. This suggested that the homoeo-alleles caused by duplications of chromosome have been maintained well in this species.

Key words: inheritance, isozymes, allozymes, *Pinus thunbergii*, Japanese black pine, acrylamide gel electrophoresis.

Zusammenfassung

Bei *Pinus thunbergii* PARL. wurde die Vererbung von Isoenzym-Varianten bei 19 Enzym-Systemen mittels Polyacrylamid-Gel-Elektrophorese untersucht. Bei der Analyse haploider Megagametophyten-Gewebe zeigte sich, daß Allozymvarianten bei diesen Enzymen durch insgesamt 120 Strukturgene an 37 Loci kodiert werden. An fünf dieser Loci, Adh-2, Me-2, T20-3, T20-4 und Got-3 wurde keine Variation festgestellt. Bei den übrigen 32 Loci wurden mehr als 2 Varianten an jedem Locus gefunden. Die Bestätigung der 1:1 Aufspaltung in Saatgut heterozygoter Bäume zeigt, daß diese Allozyme den Mendelschen Gesetzen unterliegen. Drei Gruppen von Isoenzymen wurden beobachtet, die gleiche elektrophoretische Merkmale aufwiesen, jedoch von unterschiedlichen Genloci kodiert wurden. Dies deutet an, daß Homöo-Allele, die durch Chromosomenverdopplung entstehen, bei dieser Art vorliegen.

Introduction

Recently, isozyme techniques have been widely used in genetic studies of forest trees. They have contributed to various aspects in the practical forest tree breeding, that is (1) to identify the clone, (2) to judge the place of the seeds' origin, (3) to establish the managing skills in seed orchards, (4) to know the genetic structure in natural forest population and genetic difference between natural populations in order to manage the natural forest, and to conserve the gene resources.

The most effective use should be expected when applying the isozyme as a marker gene to grasp the genetic variation. Therefore, it is necessary to account for the inheritance of isozymes. The more markers we found, the more reliable results are expected and also, the more suitable markers to each research aim are available. Genetic analysis of isozyme has been improved rapidly using megagametophyte chiefly in conifer since BARTELS (1971) utilized it.

Genetic analysis has been carried out and a lot of loci were found in each gymnospermous species especially in *Pinus ponderosa* (O'MALLEY *et al.*, 1979; MITTON *et al.*, 1979), *P. sylvestris* (RUDIN and RASMUSON, 1973; RUDIN, 1975 and 1977; RUDIN and EKBERG, 1978), *P. contorta* (WHEELER and GURIES, 1982; DANCIK and YEH, 1983), *P. banksiana* (DANCIK and YEH, 1983), *P. taeda* (ADAMS and JOLY, 1980), *P. rigida* (GURIES and LEDIG, 1978), *P. strobus* (ECKERT *et al.*, 1981), *Pseudotsuga menziesii* (YEH and O'MALLEY, 1980), and *Picea glauca* (KING and DANCIK, 1983).

This paper describes the inheritance of 19 enzyme systems detected from megagametophyte tissue in Japanese black pine (*Pinus thunbergii* PARL.).