this family and it seems the optimal in respect of chromosomal sets (genomes) has been reached at the diploid level.

Zusammenfassung

23 Arten und 10 Varietäten aus dem Himalaja und angrenzenden Gebieten wurden untersucht, davon 18 Arten und 10 Varietäten erstmalig. Alle sind diploid (2n = 24 Chromosomen) und zeigen mit Ausnahme einer Population von *Castanopsis* tribuloides eine normale Meiose. Morphologische Variabilität fand sich bei Quercus lamellosa, Q. lineata var. lobbii, Pasania *pachyphylla* und Castanopsis tribuloides. Polyploide gibt es in der Familie der Fagaceae nur selten.

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A Chromatographic Study of Foliage Polyphenols in Pine Hybrids (Subsection Sylvestres)[1])

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The application of paper and thin-layer chromatography of phenolic compounds has been especially useful in plant genetics research. A series of studies on interspecific hybridization in Baptisia showed that species-specific compounds were of use in evaluating hybrid populations involving up to four species (ALSTON and TURNER, 1963a), that the cumulative inheritance of flavonoid compounds was consistent in the hybrids (ALSTON and HEMPEL, 1964), and that chromatography more clearly defined the structure of hybrid swarms than did morphological characteristics (MCHALE and ALSTON, 1964). In forest tree species, chromatographic techniques have been used for identifying hybrids in Betula (CLAUSEN, 1962, 1963; DUGLE, 1966), *Prunus* (HASEGAWA and SHIRATO, 1963), and Picea (HANOVER and WILKINSON, 1970). HOFF (1968) reported on the identification of the hybrid of Pinus monticola and P. flexilis by paper chromatography of foliage polyphenols.

In an earlier study (THIELGES, 1969), the interspecific relationships among species of the Subsection *Sylvestres* LOUD. of the genus Pinus were investigated by two-dimensional paper chromatography of foliage polyphenols. Through the analyses of large numbers of individual tree samples collected from populations located throughout the range of each species, discrete and consistent chromatographic patterns were established for each of the taxa included in the study. These results provided the basis for the present study on the inheritance of foliage polyphenols and the evaluation of this chromatographic technique for the verification of interspecific hybrids in *Sylvestres*.

Materials and Methods

Foliage samples of artificial hybrid progenies representing several species combinations were collected from test plantings at Norfolk, Connecticut, Washington's Crossing, New Jersey, and Maple, Ontario, Canada. In addition, samples of natural hybrids were supplied by collectors in Canada and France. Reciprocal crosses, backcrosses, and F_1 progenies were included in the study. Information on these progenies is supplied in Table 1.

Preliminary experiments indicated qualitative differences in foliage polyphenol content due to foliage age and season of collection. Therefore, only year-old (1965 growth) foliage was sampled and all collections were made in June and July, 1966. Prior analyses of samples collected from natural stands and replicated plantings of the same geographic seed sources revealed quantitative but not qualitative variation in polyphenol content (THIELGES, 1968). This

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Table 1. — Progenies of interspecific hybrids analyzed by chromatography of polyphenols.

Species Combination	Artificial (A) or Natural (N) Hybrids	No. of Trees	Foliage Collection Site
P. sylvestris × *nigra*	(A)	4	Washington's Crossing, N. J.
P. nigra × *sylvestris*	(A)	9	Maple, Ontario
P. mugo × *sylvestris*	(N)	7	Nancy, France
Backcross to *P. sylvestris*	(A)	4	Maple, Ontario
P. densiflora × *sylvestris*	(A)	9	Washington's Crossing, N. J.
P. densiflora × *nigra*	(A)	5	Maple, Ontario
P. nigra ×*densiflora*	(A)	5	Maple, Ontario; Norfolk, Ct.
(*P. densiflora* × *nigra*) × *nigra*	(A)	5	Maple, Ontario
(*P. densiflora* × *nigra*) F_2 (selfed)	(A)	4	Maple, Ontario
P. densiflora × *thunbergiana*	(N)	5	Maple, Ontario
P. densiflora × *thunbergiana*	(A)	4	Norfolk, Ct.
Backcross to *P. densiflora*	(A)	6	Norfolk, Ct.
Backcross to *P. thunbergiana*	(A)	8	Norfolk, Ct.
P. thunbergiana × *densiflora*	(A)	12	Norfolk, Ct.
P. nigra × *thunbergiana*	(A)	5	Norfolk, Ct.
P. thunbergiana × *nigra*	(A)	5	Washington's Crossing, N. J.

lack of environmental variation allowed valid chromatographic comparisons to be made among samples collected from diverse sites.

Prior to analysis foliage samples were dried at 45° C for 72 hr. Eight g of dried tissue was homogenized in 100 ml of ethyl ether for 4 min in a Waring semi-micro Blendor. The homogenized mixture was transferred to a 250-ml Erlenmeyer flask and placed on a rotary motion shaker. Three decantings with 50 ml of fresh ether at 12-hr intervals extracted all ether-soluble compounds.

The tissue was then extracted with n-butanol by the same procedure for 48 hr. The n-butanol fractions, containing polyphenols, were evaporated to dryness on a Buchler Rotary Evapomix and redissolved in 1.0 ml of n-butanol prior to chromatography. Compounds were separated on Whatman 3 MM filter paper (46 × 57-cm sheets) by two-dimensional descending chromatography. Extracts were applied with a micropipet in 10-μliter increments to a total of 120 μliters.

Table 2. — Chromatographic data on polyphenolic constituents in foliage of *Pinus* (subsection *Sylvestres*) hybrids.

Compound Number	Rf		UV Color		Color Sulfanilic Acid-Na_2CO_3 Spray
	(BAW)	($HCOON_a$)	Untreated	NH_3	
1	.14	.00	Y	Y	—
9	.23	.02	B	B	—
12	.24	.18	Y	Y	—
13	.24	.33	Y	Y	Y
17	.25	.75	LB	LB	—
29	.32	.07	Y	Y	—
34	.33	.92	—	—	Br
37	.34	.44	Y	Y	—
41	.37	.08	Y	Y	—
43	.38	.43	Y	Y	Y
52	.43	.88	LV	LV	—
54	.45	.07	Y	Y	—
55	.46	.08	B	B	—
61	.47	.91	—	R	D Br
64	.48	.51	Y	Y	Y
68	.51	.51	BG	BG	—
*75	.55	.32	LB	LB	Y
79	.56	.67	BG	BG	—
83	.58	.48	Y	Y	Y
85	.59	.17	DV	DV	—
86	.60	.79	—	—	Y
87	.61	.56	LB	LB	—
88	.61	.72	B	B	—
89	.62	.03	DV	DV	—
92	.63	.14	B	B	—
93	.64	.62	Y	Y	Y
97	.69	.22	DV	DV	Br
100	.70	.29	Y	Y	—
111	.76	.51	—	—	R Br
113	.79	.55	—	—	Y

* Identified as D-catechin by co-chromatography.
Color abbreviation: B = blue; LB = light blue; BG = blue-green; Br = brown; D Br = dark brown; R Br = red-brown; R = red; Y = yellow; LV = light violet; DV = dark violet.

The solvent combination employed was the organic (upper) phase of n-butanol : acetic acid : water (4 : 1 : 5 v/v) followed by an aqueous solution of sodium formate (10 g HCOONa : 200 ml H_2O : 2 ml formic acid). Development times were approximately 18 hr and 4.5 hr, respectively. Compounds were located by viewing the chromatograms under ultraviolet light before and after exposure to ammonia vapors and by color reactions when sprayed with diazotized sulphanilic acid reagent followed by a 10% solution of sodium carbonate in water.

Compounds were classified by the R_f values of spots and by ultraviolet fluorescence and spray reagent color reactions. Each discrete spot was assigned a number. Only one compound (No. 75, D-catechin) was identified; it serves as a reference for the other spots. Data for the compounds discussed in this study are presented in *Table 2.*

The data were analyzed on the basis of total chromatographic affinity of the hybrids with the parent species (progeny affinities) and also from the standpoint of the inheritance of individual compounds. These two aspects of the study are discussed separately, below.

Results and Discussion

Chromatographic Affinities: — By regarding each compound as a separate character, individual hybrids were compared with each parent species by the application of the "paired affinity (PA)" analysis of Ellison, Alston, and Turner (1962);

$$\text{"PA"} = \frac{\text{Compounds in common for hybrid + parent}}{\text{total compounds in hybrid + parent}} \times 100.$$

The results of these comparisons are shown in *Table 3* in terms of the average affinities of the hybrid progenies with each parent. The similarities of individual hybrids to each parent are also presented. "Intermediate" hybrids were those which possessed chromatographic affinity of approximately the same magnitude to both parent species.

In general, the hybrids contained all or most of the compounds common to both parent species. The differences in mean affinity values indicate the degree to which the hybrids contained compounds specific to one or the other parent. In no instances did the chromatographic pattern of a hybrid represent the summation of the patterns of both parent species.

The effect of backcrossing on the chromatographic patterns of the hybrids is illustrated in *Table 4.* A comparison of progeny affinities shows that in all cases, backcrossing resulted in an increase in chemical affinity with the recombinant species. This was sometimes accompanied by a decrease in chemical affinity with the non-recombinant species. These results suggest that multiple-factor inheritance is involved in the synthesis of most compounds.

The results of the progeny affinity analyses indicate that chromatography of polyphenols can be used for identifying *Pinus* interspecific hybrids when the chromatographic patterns of the parent species have been determined.

Inheritance of individual compounds: — The investigation of inheritance patterns of individual compounds was somewhat limited because of small numbers of F_1 and backcross progeny and the lack of F_2 progeny. However, the species combinations of *P. densiflora* × *nigra, P. mugo* × *sylvestris,* and *P. densiflora* × *thunbergiana* provided adequate data for genetic analyses. In the following discussion, hypotheses based on observations of the occurrence of several unknown compounds in different mating systems are presented.

Zeijlemaker and Mackenzie (1966) reported that the inheritance of a methoxy flavonoid compound in the leaves of *Acacia mearnsii* is apparently controlled by a single dominant gene. In the present study, compounds 88 and 92 conformed closely to a monogenic inheritance pattern with complete dominance. The two compounds were consistently present in all of the parent species except *P. densiflora.* It

Table 3. — Affinities of chromatographic patterns of interspecific F_1 hybrids with parent species patterns.

Species Combination	$\bar{x}$ Affinity		Individual affinities to		
	female	male	female	intermediate	male
P. sylvestris × *nigra*	82.5	76.8	3	1	0
P. nigra × *sylvestris*	81.1	76.5	7	1	1
P. mugo × *sylvestris*	90.2	75.7	7	0	0
P. densiflora × *sylvestris*	76.8	72.1	8	0	1
P. densiflora × *nigra*	68.9	69.5	3	1	1
P. nigra × *densiflora*	78.6	71.7	5	0	0
P. densiflora × *thunbergiana*	76.0	70.9	7	1	1
P. thunbergiana × *densiflora*	79.0	76.4	6	2	4
P. nigra × *thunbergiana*	81.8	73.9	4	1	0
P. thunbergiana × *nigra*	78.2	80.7	2	0	3

Table 4. — Effect of backcrossing on *Pinus* progeny affinities.

Species Combination	$\bar{x}$ Affinity to		Individual Affinities		
	densiflora	*thunbergiana*	*densiflora*	*Int.*	*thunbergiana*
1. *P. densiflora* × *thunbergiana*	76.0	70.9	7	1	1
Backcross to *P. densiflora*	79.5	71.9	3	2	1
Backcross to *P. thunbergiana*	76.0	87.0	0	0	8
	sylvestris	*mugo*	*sylvestris*	*Int.*	*mugo*
2. *P. mugo* × *sylvestris*	75.7	90.2	0	0	7
Backcross to *P. sylvestris*	83.4	82.8	2	1	1
	nigra	*densiflora*	*nigra*	*Int.*	*densiflora*
3. *P. densiflora* × *nigra*	69.5	68.9	1	1	3
Backcross to *P. nigra*	73.2	73.4	1	1	3

Table 5. — Inheritance pattern for compound 88. Monogenic inheritance — complete dominance.

Species Combination and Genotype	Genotypic Ratio	Phenotypic Ratio			
		Observed		Expected	
		With 88	Without 88	With 88	Without 88
P. densiflora × *nigra* (aa) × (AA)	A11 Aa	10	0	10	0
Backcross to *P. nigra* (Aa) × (AA)	3AA : 1Aa	5	0	5	0
F_2 (Selfed) (Aa) × (Aa)	1AA : 2Aa : 1aa	3	1	3	1
P. thunbergiana × *densiflora* (AA) × (aa)	A11 Aa	19	2	21	0
Backcross to *P. densiflora* (aa) × (Aa)	2Aa : 2aa	4	2	3	3
Backcross to *P. thunbergiana* (AA) × (Aa)	3AA : 1Aa	7	1	8	0

Table 6. — Inheritance pattern for compound 92. Monogenic inheritance — complete dominance.

Species Combination and Genotype	Genotypic Ratio	Phenotypic Ratio			
		Observed		Expected	
		With 92	Without 92	With 92	Without 92
P. nigra × *densiflora* (BB) × (bb)	A11 Bb	9	1	10	0
Backcross to *P. nigra* (Bb) × (BB)	3BB : 1Bb	5	0	5	0
F_2 (Selfed) (Bb) × (Bb)	1BB : 2Bb : 1bb	3	1	3	1
P. thunbergiana × *densiflora* (BB) × (bb)	A11 Bb	20	1	21	0
Backcross to *P. thunbergiana* (Bb) × (BB)	3BB : 1Bb	8	0	8	0
Backcross to *P. densiflora* (Bb) × (bb)	2Bb : 2bb	3	3	3	3

was assumed that *P. densiflora* is a homozygous recessive and all other species in the study are homozygous dominants for the single genes controlling the synthesis of each compound. The results of these analyses are presented in *Tables 5 and 6.* In addition to the species combinations included in these tables, other crosses that involved homozygous dominant species supported the hypothesis, as compounds 88 and 92 were present in all hybrids. To further test the hypothesis of monogenic control of these two compounds, however, the progeny of a cross involving two homozygous recessive species should be analyzed.

The rather small number of progenies and limited backcross and F_2 material made it even more difficult to determine distinct multigenic inheritance patterns, but it was evident that the synthesis of most of the polyphenols in *Pinus* foliage is controlled by more than one gene. The general absence of discrete ratios indicated that nonallelic gene interactions are involved and also that dominance may not be complete in many cases.

Traits exhibiting a discontinuously segregating distribution and a multigenic inheritance are termed threshold characters or "all or none" traits. Expression is dependent on multiple additive effects. The concept of threshold characters was first introduced by Sewall Wright (1922) to explain the results of polydactyly in guinea pigs. Gruneberg (1952) used the term "quasi-continuous" inheritance in a study of skeletal traits in mice. Genetic studies of threshold inheritance involve the observation of the effects of backcrossing on the frequency of the trait in the population.

In the hybrid *Pinus* progenies of this study, backcrossing resulted in an increase in the frequency of compounds specific to the recombinant parent and a decrease in the frequency of those compounds specific to the nonrecombinant parent *(Tables 7—9).* This is especially evident in *Table 7,* where a relatively large number of F_1 progeny were analyzed and the progenies of the backcross to both parents were available. Apparently, the synthesis of these compounds requires a certain number or proportion of active alleles at a "threshold" level. This situation is reflected to some degree by the changes in total chemical affinity in backcross progenies *(Table 4).*

Individual compounds followed the same general frequency pattern in all of the species combinations *(Tables 7—9).* Moreover, the magnitude of the frequencies of *P. densiflora* compounds 37, 55, and 85 are essentially the same in combination with *P. thunbergiana (Table 7)* and *P. nigra (Table 9).*

The relative change in frequency of the compounds with backcrossing may be an indication of the number of genes involved in their synthesis. For example, in *Table 7,* the relatively large changes in the frequency of compounds 13, 41, 55, and 89 may indicate a fewer number of genes involved in the synthesis of these compounds than for compounds 37, 43, and 83 which exhibited minor changes with

Table 7. — Threshold inheritance (multiple additive effects) of polyphenols. *Pinus densiflora* × *thunbergiana.*

	Compound Number	Frequency of Compound (%)		
		F_1	Backcross to *P. densiflora*	Backcross to *P. thunbergiana*
P. densiflora	9	9.5	66.7	0.0
Compounds	13	38.1	66.7	12.5
	17	9.5	50.0	0.0
	37	14.3	16.7	12.5
	41	42.9	83.3	0.0
	43	4.8	16.7	0.0
	55	19.0	66.7	0.0
	75	66.7	66.7	37.5
	79	14.3	66.7	12.5
	83	14.3	16.7	0.0
	85	14.3	50.0	0.0
	89	33.3	66.7	16.7
P. thunbergiana	29	14.3	0.0	50.0
Compounds	34	19.0	16.7	25.0
	86	42.9	0.0	50.0
	93	52.4	33.3	75.0

Table 8. — Threshold inheritance (multiple additive effects) of polyphenols. *Pinus mugo* × *sylvestris.*

	Compound Number	Frequency of Compound (%)	
		F_1	Backcross to *P. sylvestris*
P. sylvestris	1	0.0	25.0
Compounds	12	28.6	50.0
	68	0.0	100.0
	87	14.3	100.0
	89	57.1	100.0
P. mugo	43	28.6	0.0
Compounds	52	71.4	25.0
	61	85.7	0.0
	64	100.0	0.0
	97	28.6	0.0
	100	14.3	0.0
	111	71.4	50.0
	113	42.9	0.0

Table 9. — Threshold inheritance (multiple additive effects) of polyphenols. *Pinus densiflora* × *nigra.*

	Compound Number	Frequency of Compound (%)	
		F_1	Backcross to *P. nigra*
P. densiflora	9	60.0	0.0
Compounds	37	20.0	0.0
	55	20.0	0.0
	85	30.0	0.0
P. nigra Compound	54	30.0	60.0

backcrossing. With more backcross generations, larger progenies, and more data on the biosynthesis of identified compounds, it should be possible to determine the number of active alleles involved in the synthesis of individual compounds.

Almost every hybrid was found to contain compounds not present in either parent species. Some of these were characteristic of other *Sylvestres* species, but a few were "new" compounds. The occurrence of these latter compounds is evidence of chemical interaction above the gene level. Alston and Turner (1963 b) have provided a discussion of the genetic basis for the production of "hybrid substances".

The occurrence of "hybrid" compounds in these *Pinus* progenies is probably the result of complementary gene interactions of varying degree. It is also probable that modifier systems may be operative resulting in differences in penetrance and expressivity. The same "hybrid" compounds often occur in the F_1 progeny of several different species combinations. This indicates a degree of genetic homogeneity among *Sylvestres* species of a polygenic system that may have been modified through evolutionary divergence.

It must be stressed that the results and hypotheses discussed above are based on the analyses of rather limited genetic material. Further investigation of the relationships reported herein will depend on the production of F_2 and further backcross progenies and analyses of this material. Also, while the chromatographic techniques employed in this study provided a means of fairly rapid and reliable analysis of large numbers of samples, it is obvious that "unknown" compounds can provide only a limited amount of genetic information. Further genetic studies would be greatly facilitated by the identification of compounds (at least as to class) and information regarding their synthesis including relationships among the various compounds produced in any individual.

In a selection and breeding program, chromatographic analysis of secondary compounds such as phenols and terpenes may be useful as genetic markers, especially if they are shown to be linked with economically desirable characteristics. For example, Hanover (1966) found monoterpene levels in *Pinus monticola* to be under strong genetic control. Significant negative correlations of alpha-pinene and total terpene content with growth suggested the possibility of indirect selection for growth rate.

It is possible that phenolic compounds may also be important physiological factors determining incompatibility among *Sylvestres* species. McWilliam (1959) suggested a chemical basis for incompatibility among these species in the form of a pollen tube growth factor. There is a growing body of literature concerning auxin-phenol interactions (Shantz, 1966) and the findings of Strohl and Seikel (1965) indicate considerable interspecific variation in the phenols of *Pinus* pollen. Ching, Aft, and Highley (1965) identified different flavonoids in floral phenotypes of *Pseudotsuga menziesii* that might be related to incompatibility between these phenotypes. These findings suggest the possibility of expanding the present study to include analyses of reproductive tissues of *Pinus.*

Summary

Paper-chromatographic analyses of polyphenolic compounds in foliage provided a means of studying interspecific hybrids in the Subsection *Sylvestres* that is adaptable to breeding programs and to problems of natural hybridization and introgression. Chromatographic patterns of F_1 hybrids generally contained all of the compounds common to both parent species and varying numbers of species-specific compounds. In no instances were the hybrid patterns a summation of the patterns of the parent species. Compounds not present in either parent species were constituents of many hybrids. Some of these "hybrid" compounds were characteristic of other *Sylvestres* species. This indicates some degree of genetic homogeneity of a polygenic system for phenolic synthesis within this subsection. Two compounds followed a pattern of monogenic inheritance, although a system of cumulative additive effects or "threshold inheritance" seems to govern the synthesis of most of the phenols in *Pinus* foliage. This latter hypothesis was based on the results of analyses of the frequency of individual compounds of F_1 and backcross progenies and by observation of the changes in hybrid-parent chromatographic affinity in these progenies.

Zusammenfassung

Papierchromatographische Analysen von Polyphenol-Verbindungen an Nadeln von Artbastarden der Subsektion *Sylvestres* erwiesen sich für die Anwendung bei Züchtungsprogrammen und anderen Untersuchungen geeignet. Die chromatographischen Muster der F_1 enthalten im allgemeinen alle Substanzen, die beiden Elternarten gemeinsam sind. Bei keinem Besipiel war aber das Hybridmuster eine einfache Summierung der Muster beider Elternarten. Verbindungen, die in einem der Elternarten nicht vorhanden waren, kamen aber bei vielen Bastarden vor. Diese „Bastard"-Verbindungen waren wiederum charakteristisch für andere *Sylvestres*-Arten. Dies weist auf eine gewisse genetische Homogenität eines polygenen Systems der Phenolsynthese innerhalb der Subsektion hin.

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Intraspecific Variation in Foliage Polyphenols[1]) of Pinus (Subsection Sylvestres)

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The use of chemical characters in systematic studies is not new, but the development and refinement of chromatographic methods have greatly aided the study of the distribution of natural organic compounds within and among plant taxa. In the genetic approach to plant taxonomy, useful information has been obtained through studies of the secondary, or intermediate, metabolites of plants such as terpenes, alkaloids, and phenols.

Phenolic compounds are especially well-suited to empirical surveys of variation at lower taxonomic levels because of their diverse chemical and physical properties and the rather restricted taxonomic distribution of individual compounds. One explanation for the extreme variability in the distribution of plant phenols is that they have evolved more recently than basic metabolites and have not been subjected to such rigorous selection pressures as sugars, amino-acids, and organic acids. But the physiological functions of most phenolic compounds are either unknown or disputed and this makes difficult the development of theories to explain the selective advantage of individual compounds.

Research on the action of plant phenols as phytoalexins in diseased plant tissue (Cruickshank and Perrin, 1964; Hare, 1966) and as regulators and inhibitors of plant growth (Shantz, 1966) indicates that many of these compounds are physiologically active.

Regardless of whether the genetic mechanism responsible for this variation is natural selection, random genetic drift, or mutation with subsequent reproductive isolation, plant phenols are potentially useful for studies of natural variation.

While there has been much interest in the phenolic constituents of wood, particularly their role in lignin formation and the inhibition of fungi and bacteria, there have been few systematic studies utilizing the phenolic compounds in the foliage of forest trees. Qualitative intraspecific variation in phenolic compounds from the foliage of *Picea, Salix,* and *Populus* was reported by Börtitz (1962,

[1]) This work represents a portion of a dissertation presented to the Faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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