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Chemical Relationships between *Pinaceae*

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Key Word Index – *Abies; Cedrus; Keteleeria; Larix; Picea; Pinus; Pseudolarix; Pseudotsuga; Tsuga;* Pinaceae; flavonoids; chemotaxonomy.

Abstract – Fingerprints of phenolic compounds of leaf extracts of eleven pine species have been made by paper chromatography and HPLC. The results suggest a chemical relationship which agrees fairly well with those based on immunological and morphological characters but not always with the classification commonly used.

Introduction

Three subfamilies of the Pinaceae are distinguished on the basis of their shoot systems: the Abietoideae (*Abies, Cathaya, Keteleeria, Picea, Pseudotsuga* and *Tsuga*), the Laricoideae (*Cedrus, Larix* and *Pseudolarix*) and the Pinoideae (*Pinus*) [1, 2]. The relationships between the genera of the family have been treated by several taxonomists resulting in contradictory views. For example, Flous [3] proposed a phylogenetic relation based on morphological and fossil evidence in which *Larix* and *Pseudotsuga,* or *Cedrus* and *Abies* are considered to be more closely related to each other than to the species of their respective subfamilies. Doyle [4] investigated pollination mechanisms in the Pinaceae, and distinguished two lines, one comprising of *Pinus, Picea, Larix* and *Pseudotsuga* and the second of *Pseudolarix? Abies, Cedrus* and *Tsuga*. From immunological data Prager *et al.* [5] proposed that *Larix* is more closely related to *Pseudotsuga* than to *Cedrus.*

Our work on leaf phenolics of the Pinaceae [6–9] indicates that there is a close relationship between all *Larix* species [6], some similarity between *Larix* and *Cedrus* [8] and large differences between *Larix* and *Pseudolarix.* Therefore, more genera of the Pinaceae were examined using flavonoid "fingerprints".

Results and discussion

The distribution of flavonoids extracted from needles of eleven species of nine genera of the Pinaceae is given in Table 1. Identification of the compounds is based on colour reactions and *R*$_f$

TABLE 1. OCCURRENCE OF SOME REFERENCE COMPOUNDS FROM PAPER CHROMATOGRAPHIC FINGERPRINTS OF NEEDLE EXTRACTS OF PINACEAE

		Compound*									
No.	Species	MG	LG	SG	QGa	KG/IG	KCG	V/GV	UV-i.b.	DNA-p	Comments
1.	*Abies balsamea* (L.) Mill.	+	?		±	+	+		++	+	Flavonoids covered by blue fluorescent compounds
2.	*Abies grandis* (Dougl.) Lindl.	+	+	±	+	±	+			+	
3.	*Cedrus libani* A.Rich. in Bory	+	+	+	+	?	+			±	
4.	*Keteleeria fortunei* (A.Murr.) Carr.	±	?	?	–	–	–			+	Mainly FR-gr colouring compounds
5.	*Larix sibirica* Ledeb.	+	±	±	+	±	+	++		±	
6.	*Picea breweriana* S.Watson	±?	?	+	±		±		++	+	As No.1.
7.	*Pinus jeffreyi* Grev. & Balf.	±	±	±	±	+	+			+++	
8.	*P. silvestris* L.	±	±	±	±	±?	+			+++	
9.	*Pseudolarix amabilis* (J.Nels) Rehd.	++	?	?	±	?	±			–	
10.	*Pseudotsuga mensiezii* (Mirbel) Franco	+	+	±	++	+	+	+		+	
11.	*Tsuga chinensis* (Franch.) Britzel	+	+	–	+	+	++			+	

*Reference compounds: MG = myricetin-3-glucoside, LG = laricitrin-3-glucoside, SG = syringetin-3-glucoside, KG = kaempferol-3-glucoside, IG = isorhamnetin-3-glucoside, KCG = kaempferol-3-(*p*-coumarylglucoside), V = vitexin, GV = glycosylvitexin. Spray reagents: DNA = diazotized *p*-nitroaniline, FR = flavone reagent. Colours: i.b. = intense blue, p = purple, gr = green (colour with FR obtained with flavones of flavonols with one hydroxyl group in the B-ring). ++ = abundantly present, + = present, ± = comparatively low concentration, ? = spot present with same R_f but probably not identical. – = no spot with similar R_f.

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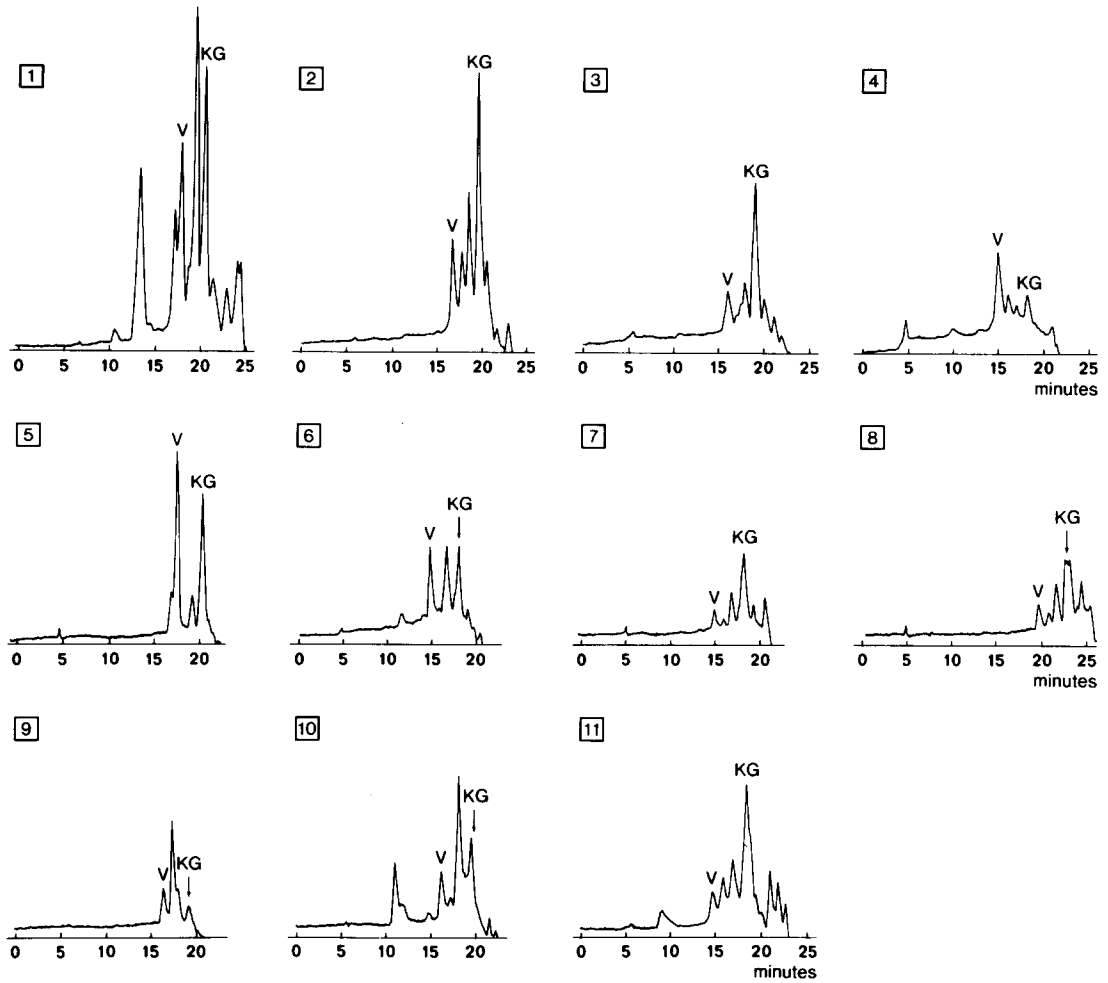

FIG. 1. HPLC ANALYSIS OF THE BUTANOL EXTRACTS OF NEEDLES OF THE PINACEAE ON ZORBAX ODS ELUTED WITH A GRADIENT OF AQUEOUS METHANOL WITH 0.1% OF PHOSPHORIC ACID. 1. *Abies balsamea*, 2. *Abies grandis*, 3. *Cedrus libani*, 4. *Keteleeria fortunei*, 5. *Larix sibirica*, 6. *Picea breweriana*, 7. *Pinus jeffreyi*, 8. *Pinus silvestris*, 9. *Pseudolarix amabilis*, 10. *Pseudotsuga mensiezii*, 11. *Tsuga chinensis*. Reference compounds: V = vitexin, KG = kaempferol-3-glucoside.

values using paper chromatography in three solvents, since only "fingerprint" comparisons were sought. For several species, confirmation of the identity of the flavonoids present was obtained from previous work (*Cedrus* [8], *Larix* [6], *Pinus* [9], *Pseudolarix* [7], *Abies* [10]. The HPLC chromatograms of the butanol-soluble portion of the needle extracts are shown in Fig. 1.

From both PC and HPLC data two general facts are apparent. First, the needles of all species investigated are comparatively rich in flavonoids. Second, the species examined show a more or less similar pattern, except for *Keteleeria fortunei* and *Pseudolarix amabilis*. The latter has one main flavonoid, previously identified as myricetin-3-rhamnoside [7] and, in contrast to other species, acylated flavonoid glycosides are more or less absent. *K. fortunei*, on the other hand, has a large number of flavonoids giving a green colour with flavone reagent [12] indicating mono-hydroxylation of the B-ring. Both *Pinus* species were marked by a high number of compounds giving a purple colour with diazotized *p*-nitroaniline (lignans?).

Abies balsamea and *Picea breweriana* were difficult to compare in terms of their flavonoid content by PC because of the occurrence of several intense

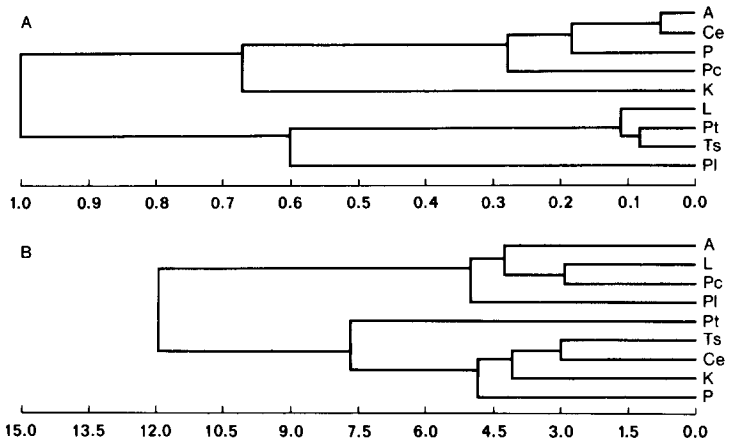

FIG. 2. RELATIONS BETWEEN PINACEAE SPECIES. A: Derived from paper chromatographic fingerprints. B: from HPLC. For abbreviations of names of plant species, see Table 2.

blue fluorescent compounds (acetophenones or stilbenoids?) which overlapped the area where most flavonoids occur.

The results from both PC and HPLC fingerprints were subjected to numerical pattern analysis (Fig. 2). In this respect HPLC by itself proved to be inadequate. This is because many flavonoids, several of which were found in the extracts investigated [13], have similar retention times on the HPLC system used [14] and thus cannot be differentiated using the techniques outlined here. This is especially obvious for species like *K. fortunei,* which contain a number of apigenin derivatives which co-chromatograph on HPLC with several flavonol glycosides which are present in *Larix* or *Abies.* The dendrogram obtained via PC data (Table 2, Fig. 2A) contains more information on the identity of the compounds and therefore gives a better representation of their differences. Some of the more striking aspects seen in these data are the close similarities found between *Cedrus* (Laricoideae) and *Abies* (Abietoideae), and between *Larix* (Laricoideae) and *Pseudotsuga* and *Tsuga* (Abietoideae), and the separate position of *Pseudolarix* (Laricoideae). Although needles of only one species of each genus have been investigated, the pattern of Fig. 2A shows a close resemblance to the phylogenetic trees based on morphological [3] and immunological [5] characters.

In Fig. 3 we have combined the data from Flous [3] and Prager *et al.* [5] with some of our PC results to show a two-dimensional representation of the phylogenetic relationship of the Pinaceae. In this figure, the distances between the genera are mainly based on Prager's antigenic distances

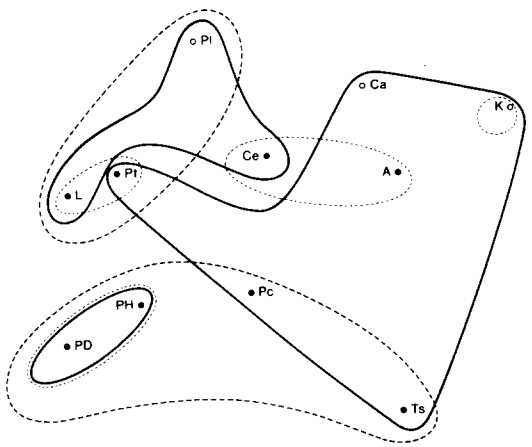

FIG. 3. RELATIONSHIPS IN PINACEAE. A = *Abies,* Ca = *Cathaya,* Ce = *Cedrus,* K = *Keteleeria,* L = *Larix,* Pc = *Pinus,* (H = *Haploxylon,* D = *Diploxylon*), Pl = *Pseudolarix,* Pt = *Pseudotsuga,* T = *Tsuga.* Distances between the species on immunological [5] and chemical data. Dotted lines represent the phylogenetic relationships according to Flous [3]. The full lines give the three subfamilies Abietoideae, Laricoideae and Pinoideae.

combined with our PC data (especially for *Pseudolarix* and *Keteleeria,* which were not investigated by Prager *et al.*). The dotted lines represent Flousphylogenetic relationship and the full lines indicate the classification into the three subfamilies. The latter classification appears rather artificial and, in view of the immunological and chemical data, a reconsideration of Flous' [3] ideas seems worthwhile.

TABLE 2. MATRIX OF SIMILARITY COEFFICIENTS (×100) OBTAINED FROM PAPER CHROMATOGRAPHIC FINGERPRINTS

	A	Ce	K	L	Pc	P	Pl	Pt	Ts
A	–	41	583	117	173	162	540	833	164
Ce		–	313	67	220	107	417	556	556
K			–	386	400	500	1100	514	344
L				–	329	211	300	80	100
Pc					–	230	900	191	230
P						–	480	136	175
Pl							–	329	580
Pt								–	68
Ts									–

A = *Abies grandis*, Ce = *Cedrus libani*, K = *Keteleeria fortunei*, L = *Larix sibirica*, Pc = *Picea breweriana*, P = *Pinus jeffreyi*, Pl = *Pseudolarix amabilis*, Pt = *Pseudotsuga mensiezii* and Ts = *Tsuga chinensis*.

Experimental

Plant material was used as in a previous investigation [11], collected from the Blijdenstein Pinetum at Hilversum. Needles were extracted with Me_2CO and, after filtration, removal of lipids with light petroleum and concentration, the residue was extracted with Et_2O. The resulting aqueous phase was re-extracted with *n*-BuOH. Both the ether and the butanol extracts were further investigated by paper chromatography (PC) and by high-performance liquid chromatography (HPLC). For PC comparison three solvents were used 15% HOAc, tert. BuOH–HOAc–H_2O (3:1:1) and water-saturated phenol. The sheets were examined under UV with and without NH_3. Afterwards they were sprayed with flavone reagent [12] or with diazotized *p*-nitroaniline followed by sodium carbonate (DNA).

HPLC's were made with a Dupont 830 chromatograph with a 4.6 i.d. × 240 mm Zorbax ODS column at 50°, eluted with a gradient (45–100%, concave 2) of MeOH in H_2O with 0.1% of phosphoric acid at a rate of 3%/min and a pressure of 1100–1300 psi (7500–9000 kPa, flow *ca* 0.5 ml/min).

Reference substances for PC were kaempferol-3-glucoside (KG), quercetin-3-galactoside (QGa), isorhamnetin-3-glucoside (IG), myricetin-3-glucoside (MG), laricitrin-3-glucoside (LG), syringetin-3-glucoside (SG), vitexin (V) and kaempferol-3-(*p*-coumarylglucoside) KCG). QGa was a gift from Tom Mabry, Austin; V was purchased from Roth, and the other compounds had previously been isolated from larch species. For HPLC at least two additional runs were made with V, KG or KCG as internal standards.

Similarity coefficients from the PC fingerprints were obtained from the ratio between the number of different and identical spots (Table 2). From the HPLC chromatograms percentages of peak contribution were calculated (Table 3). Numerical pattern analysis was performed by "Biopat" program system for biological pattern analysis [15]. A dendrogram was also constructed according to the method of Fitch and Margoliash [16], which gave a similar figure to that obtained by "Biopat".

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TABLE 3. RELATIVE CONTRIBUTION (IN %) OF HPLC PEAKS OF BOTH THE ETHER AND BUTANOL EXTRACTS OF PINACEAE SPECIES

	Peak No.															
Species	1	2	3 V	4	5	6	7 KG	8	9	10	11 KCG	12	13	14	15	16
Abies balsamea	7.8	8.3	7.6	5.1	15.8	–	18.9	12.7	10.7	3.6	8.7	–	1.0	–	–	–
A. grandis	–	1.2	8.0	9.5	13.5	–	31.9	18.7	11.0	2.5	2.5	1.2	–	–	–	–
Cedrus libani	–	0.6	4.4	0.6	5.9	1.6	17.5	12.5	10.9	8.1	14.3	8.8	3.8	10.9	–	–
Keteleeria fortunei	–	1.5	11.8	8.8	6.9	–	9.8	3.4	11.3	–	13.7	11.3	4.9	11.3	2.9	2.5
Larix sibirica	–	5.5	21.5	0.5	6.4	–	29.7	13.7	7.3	–	7.3	3.2	1.4	–	3.7	–
Picea breweriana	4.0	1.1	13.2	–	14.9	–	36.8	10.9	6.3	1.1	8.0	1.1	1.1	–	1.1	–
Pinus jeffreyi	–	–	4.8	4.2	7.8	–	16.9	12.7	18.7	–	28.3	–	5.4	–	1.2	–
P. silvestris	–	–	4.5	3.6	8.0	17.9	13.8	15.2	17.9	–	18.7	–	0.4	–	–	–
Pseudolarix amabilis	–	1.9	30.8	5.8	23.7	–	23.7	4.5	7.7	–	1.9	–	–	–	–	–
Pseudotsuga mensiezii	7.0	2.9	4.1	3.8	17.6	5.3	13.2	6.5	6.2	8.8	14.7	3.8	6.2	–	–	–
Tsuga chinensis	2.7	–	5.1	7.9	9.8	–	24.2	5.6	11.2	11.2	13.9	7.9	3.3	–	–	–