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STUDIES ON PLANT CUTICULAR WAXES—III.

THE LEAF WAX ALKANES AND ω-HYDROXY ACIDS OF SOME MEMBERS OF THE CUPRESSACEAE AND PINACEAE

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Abstract—Results of analyses of leaf wax alkanes and ω-hydroxy acids from members of the Cupressaceae and Pinaceae are reported and discussed as taxonomic criteria. The range of variation within a species is examined and the possibility of defining a species or species group on a chemical basis is discussed.

THE SUB-DIVISION of the conifers is the subject of considerable controversy among taxonomists. Rendle[1] recognizes six families in the order Coniferales, of which Cupressaceae and Pinaceae are two of the most extensive. Some authorities (cf. Dallimore and Jackson[2]) place the majority of the conifers in one major family, Pinaceae: others employ several orders, such as Araucariales, Podocarpales, Pinales and Cupressales to describe the group, while further authorities (cf. Harrison[3]) reduce these to families (Araucariaceae etc.). The family Cupressaceae (of Rendle[1]) has been divided into two sub-families Cupressoideae and Callitroideae: the sub-division is both a morphological and a geographical one as in general the Cupressoideae are confined to the northern hemisphere in both Eurasia and America while the Callitroideae are essentially from the southern hemisphere.

These two sub-families have been studied by Erdtman,[4] using the heartwood constituents as taxonomic criteria. In the sub-family Cupressoideae, the tribe Cupresseae contains the genus *Cupressus* which could be readily distinguished from the related genus *Chamaecyparis* by the presence of the C_{15}-tropolone nootkatin in the heartwood of the former but not of the latter. Within the genus *Cupressus* the Eurasian species were found to contain the diterpene manool which was absent from those species of American origin examined.

Of the other two tribes of the Cupressoideae, Erdtman[4] found the Thujopsideae to be a very heterogeneous group in respect of heartwood constituents. In the tribe Juniperoideae, the genus *Juniperus* showed a similarity to *Cupressus* rather than to *Chamaecyparis* both in the heartwood constituents and in the content of phenolic substances in the bark.

In the sub-family Callitroideae, the genera *Callitris* and *Neocallitropsis* had some heartwood constituents in common. Species from the southern African genus *Widdringtonia*, although lacking tropolones, showed other heartwood constituents common to the northern hemisphere Cupressoideae rather than to other members of the Callitroideae, while the sole

[1] A. B. RENDLE, *The Classification of Flowering Plants*, 2nd edition. Cambridge University Press, Cambridge (1930).

[2] W. DALLIMORE and A. B. JACKSON, *Handbook of Coniferae*, 3rd edition. Edward Arnold, London (1948),

[3] S. G. HARRISON (Revision of DALLIMORE and JACKSON[2]) *Handbook of Coniferae and Ginkgoaceae*. Edward Arnold, London (1966).

[4] H. ERDTMAN, in *Chemical Plant Taxonomy* (edited by T. SWAIN), Academic Press, London and New York (1963); H. ERDTMAN and T. NORIN, *Fortschr. Chem. Org. Naturstoffe* **24**, 206 (1966).

northern hemisphere representative of the Callitroideae, *Tetraclinis*, also showed a heartwood content similar to the Cupressoideae.

The family Pinaceae is divided by Rendle[1] into three tribes, of which the first, Pineae, contains only the genus *Pinus*, with eighty to ninety species distributed throughout the northern hemisphere. The genus has been classified by several workers,[5–7] but most authorities recognize a major sub-division into the *Haploxylon* and *Diploxylon* sub-genera. Erdtman[4] has shown that a clear chemical distinction can be made between these two sub-genera on the basis of heartwood phenolics and has further pointed out that a major difference is observable between a characteristic "Pinales chemistry" and a "Cupressales chemistry", which in the latter is characterized by the presence of tropolones in the heartwood extractives.

In continuance of our examination of cuticular waxes of leaves as possible chemotaxonomic criteria[8] we have undertaken a preliminary investigation of available species from the Cupressaceae and Pinaceae which are being grown under small-scale plantation conditions at the arboretum of the East African Agriculture and Forestry Research Organization (EAAFRO), Muguga, near Nairobi. The origin of the seed used in establishing these plots is known, and in many cases the seed is from the geographical location in which the species was first described. Shortly after the completion of the present work, Eglinton and coworkers[9] published the results of an extensive investigation of the leaf wax alkanes from a number of conifers; their particular interest in the families Podocarpaceae and Araucariaceae has meant, however, that there is virtually no overlap in the two reports.

In the Cupressaceae we have examined the leaf wax alkane fraction of a number of species drawn from the genera *Cupressus*, *Juniperus* and *Thuja* of the sub-family Cupressoideae and from the genera *Callitris* and *Widdringtonia* of the sub-family Callitroideae. the results are shown in detail in Table 1. The crude leaf waxes from these genera contain alkanes only as minor constituents (up to 15 per cent), the major fraction being a light petroleum-insoluble ester wax, the composition of which is discussed below.

A general similarity throughout the family is apparent, all the leaf wax alkane fractions having tritriacontane (C_{33}) as the dominant alkane. Within the genus *Cupressus* the differences between species are small and the gross leaf wax alkane patterns are not able to give a clear distinction between species. Differences in the order of the minor constituents are apparent, and two groups, with either C_{35} or C_{31} as the second most abundant alkane, can be distinguished. This distinction cuts right across the sub-division of the genus into Eurasian and American species (a geographical distinction which has been chemically reinforced on the basis of the heartwood content by Erdtman[4]), and therefore would not seem to be of any significant phylogenetic value. The very close similarity of the patterns from the three species *C. benthamii* Endlicher (5), *C. lusitanica* Miller (7) and *C. lindleyi* Klotzsch (8) is of interest in view of their reputed identity (cf. Ref. 3, p. 207), a problem that is studied in detail in the following paper, as also is the value of the variation in minor constituents between species as a means towards the identification of, and distinction between, species and interspecific crosses.

[5] G. R SHAW, *The Genus Pinus*, Arnold Arboretum Publication No. 5. Riverside Press, Cambridge, Mass. (1914).

[6] R. PILGER and H. MELCHIOR, in P. ENGLER, *Syllabus der Pflanzen-Familien*. Borntraeger, Berlin (1912).

[7] W. B. CRITCHFIELD and E. L. LITTLE, *Geographic Distribution of the Pines of the World*. Misc. Publ. 991, Forest Service, U.S. Dept. of Agriculture, Washington, D.C. (1966).

[8] G. A. HERBIN and P. A. ROBINS, *Phytochem.* **7**, 239 (1968).

[9] J. BORGES DEL CASTILLO, C J. W BROOKS, R. C. CAMBIE, G. EGLINTON, R. J. HAMILTON and R. PELLITT, *Phytochem.* **6**, 391 (1967)

TABLE 1. *Gymnospermae. Cupressaceae* SPECIES ALKANE FRACTIONS EXPRESSED AS A MOLE PERCENTAGE

Sub-family Cupressoideae Cupressus (Cupresseae)		C_{35}	C_{34}	C_{33}	C_{32}	C_{31}	C_{30}	C_{29}	C_{28}	C_{27}	C_{26}	C_{25}	C_{24}	C_{23}	C_{22}	C_{21}
C. funebris Endl.	(1)	26·8	3·2	54·6	0·7	3·8	0·3	8·8	0·6	1·1	0·1					
C. torulosa D. Don.	(2)	10·4	1·0	70·1	1·6	13·8	0·5	1·5	0·4	0·3	0·2	0·2				
C. torulosa (a second example)	(3)	10·0	2·6	67·5	1·5	12·7	0·6	2·3	0·5	0·6	0·4	0·4	0·6	0·3		
C. macrocarpa Hartw.	(4)	5·7	2·0	63·6	2·4	16·7	0·5	6·0	0·4	2·0	0·3	0·3	0·1			
C. benthamii Endl.	(5)	11·3	1·8	62·5	2·9	7·5	1·3	2·9	1·4	3·6	1·9	1·3	1·2	0·4		
C. arizonica Greene	(6)	7·6	1·5	74·0	1·9	10·4	0·4	1·5	0·6	1·7	0·2	0·2				
C. lusitanica Mill.	(7)	22·5	2·1	64·1	1·2	4·5	0·4	2·1	0·4	2·1	0·3	0·3				
C. lindleyi Klotzsch	(8)	19·1	2·1	61·4	1·8	9·3	1·1	2·2	0·5	1·8	0·2	0·5				
C. cashmeriana Royle ex Carr.	(9)	17·4	4·8	54·2	2·0	3·3	1·4	2·0	2·0	2·3	2·4	2·1	1·9	1·8	1·3	1·1
Juniperus (Junipereae)																
J. procera Hochst.	(10)	7·0	1·7	73·4	2·2	11·2	0·4	2·9	0·3	0·5	0·2	0·2				
J. macropoda Boissier	(11)	8·0	1·3	36·9	2·8	7·8	3·4	6·8	5·2	6·9	7·2	5·9	4·0	2·1	1·2	0·5
Thuja (Thujopsideae)																
T. occidentalis L.	(12)	17·7	3·9	46·8	2·2	1·6	2·4	3·8	3·7	3·8	3·7	3·3	2·7	1·3	0·7	0·4
Sub-family Callitroideae Callitris																
C. preissii Miq.	(13)	5·3	2·7	63·7	4·5	15·5	2·3	2·3	1·0	1·5	0·4	0·5	0·3			
C. hugelii (Carr.) Franco	(14)	6·5	2·1	80·9	1·7	6·3	0·3	1·3	0·1	0·3	0·1	0·2	0·1	0·1		
C. rhomboidea R. Br.	(15)	1·1	0·6	50·6	2·5	36·6	0·9	4·8	0·5	1·7	0·4	0·3				
Widdringtonia																
W. juniperoides Endl.	(16)	17·5	3·8	52·5	2·9	4·8	1·5	2·9	2·0	2·8	2·2	2·7	1·9	1·4	0·8	0·3
W. schwarzii Marl.	(17)	15·7	3·1	58·0	2·3	5·1	1·5	4·1	1·4	2·1	2·3	1·8	1·0	0·7	0·6	0·3
W. cupressoides Endl.	(18)	21·8	4·1	39·9	1·4	5·1	2·6	4·6	3·9	4·4	3·6	3·8	2·6	1·5	0·5	0·2
W. whytei Rendle	(19)	16·6	4·3	31·1	2·7	5·0	2·9	5·6	4·4	6·7	4·6	5·5	4·5	3·5	2·1	0·5

Of the two *Juniperus* species examined, *J. procera* Hochstetter (10) shows a typical "*Cupressus*" leaf alkane pattern and would not be distinguished from examples such as *C. arizonica* Greene (6) with which its pattern is virtually identical. *J. macropoda* Boissier (11) on the other hand shows a pattern in which the major alkane (C_{33}) is less strongly dominant and in which a wider spread of constituents, including a relatively high content of even carbon-number alkanes, is present. *Thuja occidentalis* L. (12) is similar in its alkane pattern to (11).

In the sub-family Callitroideae three examples from the genus *Callitris* and four from *Widdringtonia* were examined. Although all showed C_{33} as the major alkane, there was little other correlation. *Callitris hugelii* (Carrière) Franco (14) (probably synonymous with *C. columellaris* F. Mueller) (Ref. 3, p. 126) and *C. preissii* Miquel (13) both have a general resemblance to the "*Cupressus*" alkane pattern but *Callitris rhomboidea* R. Brown ex. L. C. Richard (15) has a higher C_{31} content than any of the other Cupressaceae examined. Of the four *Widdringtonia* species (16–19) all have C_{35} as the second most abundant alkane and all show the relatively wide spread in distribution of chain length with a significant even carbon number alkane content that was observed in *Juniperus macropoda* (11) and *Thuja occidentalis* (12) of the sub-family Cupressoideae.

To summarize, it would seem that the gross leaf wax alkane patterns in the Cupressaceae do not afford a taxonomic criterion of any great value in supporting the accepted botanical classification, in contrast to the findings of Erdtman[4] from his study of the heartwood constituents.

Table 2 shows the results obtained by gas chromatographic analysis of the alkane fraction of leaf waxes from members of the genus *Pinus*. Alkanes form less than 1 per cent of the leaf wax of most species examined, while more than 90 per cent of the wax is insoluble in light petroleum, being predominantly, as with the *Cupressus* species, an ester type wax.

Erdtman,[4] in his chemotaxonomic study of the pines, followed the classification of Shaw.[5] This classification is based on a major sub-division of the genus into the *Haploxylon* and the *Diploxylon* sub-genera, a division that was clearly confirmed by the heartwood constituents. As may be clearly seen from the data in Table 2, all the *Pinus* species examined show a wide spread in the alkane chain-length distribution with C_{27}, C_{29} or C_{31} feebly dominant only. No discrimination between *Haploxylon* and *Diploxylon* is possible, nor would identity or differentiation of species be easily demonstrated on the basis of alkane pattern. Alkane patterns in the genus *Pinus* appear to be even less discriminatory than in other families of the Gymnosperms, since Eglinton and co-workers[9] found considerable variation of pattern between species within genera from Araucariaceae and Podocarpaceae and were able to show some correlation between botanical and chemical classification. In contrast Aplin, Cambie and Rutledge[10] found the diterpene hydrocarbon content of members of the Podocarpaceae to be of little taxonomic value.

Although neither of the families Pinaceae and Cupressaceae can be sub-divided on the basis of the alkane patterns of their leaf waxes, a clear differentiation between the two families is apparent: those members of the Pinaceae examined all show a characteristically "flat" distribution pattern of alkanes, with relatively high proportions of even carbon-number members while in the Cupressaceae the predominant pattern is one with a high C_{33} content, with either C_{31} or C_{35} as the second most common constituent.

In view of the lack of clear species differentiation shown by the alkane patterns of members of the Pinaceae and the Cupressaceae, attention was turned to an examination of the

[10] R. T. APLIN, R. C. CAMBIE and R. S. RUTLEDGE, *Phytochem.* **2**, 205 (1963).

TABLE 2. *Gymnospermae. Pinus* SPECIES LEAF WAX ALKANE FRACTIONS EXPRESSED AS A MOLE PERCENTAGE

Section Haploxylon	Sub-section	Group	C_{35}	C_{34}	C_{33}	C_{32}	C_{31}	C_{30}	C_{29}	C_{28}	C_{27}	C_{26}	C_{25}	C_{24}	C_{23}	C_{22}	C_{21}
P. strobus L.	Cembra	Strobi			2·4	1·2	19·5	5·0	23·3	5·5	18·7	7·9	9·7	1·9	3·9	0·8	0·2
P. ayacahuite Ehr.	Cembra	Strobi					15·9	4·8	21·6	5·8	22·6	7·2	11·1	3·4	5·8	1·8	
Section Diploxylon	Sub-section	Group															
P. canariensis Sm.	Parapinaster	Longifoliae			3·2	2·5	8·4	5·1	11·0	10·7	13·5	15·4	15·0	8·3	4·6	1·8	0·5
P. pinea L.	Parapinaster	Pineae			13·2	7·7	19·2	9·3	15·4	8·2	12·1	6·6	3·9	2·7	1·1	0·6	
P. montezumae Lamb.	Pinaster	Australes			2·2	1·4	15·2	7·0	15·1	7·5	17·2	8·6	10·2	5·7	6·1	2·5	1·3
P. ponderosa Dougl.	Pinaster	Australes			5·4	5·1	9·3	6·8	16·1	9·5	17·2	8·4	10·8	6·1	3·5	1·4	0·4
P. occidentalis Sw.	Pinaster	Australes	0·4	0·8	7·8	5·2	20·7	8·7	16·5	8·8	12·2	7·2	5·2	3·5	2·1	0·7	0·2
P. caribaea Mor.	Pinaster	Australes		0·7	7·3	4·1	19·0	8·9	15·2	9·9	12·5	8·6	7·3	3·4	2·1	0·7	0·3
P. halepensis Mill.	Pinaster	Australes			0·6	1·8	6·2	6·0	19·9	15·0	17·8	11·0	9·2	5·6	3·5	1·8	1·6

constituents of the major fraction of the crude leaf waxes which was insoluble in light petroleum. Kariyone and co-workers,[11] in studying the leaf waxes of eighty species from thirty-two genera of the Coniferae, recognized the presence of two types of wax esters—those in which monomeric esters, formed between long-chain aliphatic alcohols and fatty acids, are the principal constituents and those in which polymeric structures, formed by the self-esterification of a series of ω-hydroxyalkanoic acids, predominate. These latter, termed estolide waxes, had earlier been shown by Bougault and Bourdier[12] to afford principally 16-hydroxyhexadecanoic and 12-hydroxydodecanoic acids on hydrolysis. Von Rudloff,[13] however, found that the leaf wax of Colorado spruce (*Picea pungens* Engelmann) contained 14-hydroxytetradecanoic acid as its principal ω-hydroxy acid, while vapour phase chromatographic analysis of the methyl esters of the acids formed on hydrolysis of the leaf wax showed also the presence of the usual series of normal alkanoic acids from lauric to stearic acid.

Von Rudloff's report indicated that for those Coniferae producing the estolide type of leaf wax, the varying proportions of the three principal ω-hydroxyalkanoic acids might afford a means of distinguishing and relating different species and thus form the basis of a chemotaxonomic classificatory system. A preliminary examination of a number of *Pinus*, *Cupressus* and *Callitris* species, for which data on leaf wax alkanes had already been obtained, was undertaken. Leaf cuticular wax samples were saponified and the acid fraction isolated: after formation of the methyl esters (and in the case of the ω-hydroxyalkanoic acids, acetylation of the hydroxyl group), analysis by gas chromatography gave the data in Table 3 (for the genus *Pinus*) and Table 8 (for genera from the Cupressaceae).

In Table 3 the molar percentage compositions of the ω-hydroxyalkanoic acid fraction and of the normal fatty acid fraction (both saturated and unsaturated) are given separately, together with the molar percentages that these two groups of compounds formed of the acid fraction as a whole. The chromatographic conditions did not permit a distinction to be drawn between different unsaturated acids of the same chain length and these are therefore reported as total unsaturated acids of a particular chain length (eg. $C_{18\ u}$).

A noticeable difference between the patterns of acids for young and old leaves from the same tree was observed (*vide* Table 3, Nos. 2a, b and 7a, b): differences in quantity of wax obtained was also observed, young green needles of *Pinus engelmannii* Carrière yielding only a fifth of the quantity of wax as an equal weight of old needles. In a similar experiment old needles of *P. ponderosa* Douglas gave 30 per cent more wax than an equal weight of young needles. Subsequent to these observations, old leaves were used routinely for all interspecific comparative work unless otherwise stated.

A sample of *Picea pungens* leaf wax, obtained from the Royal Botanic Gardens, Kew, was analysed to compare with von Rudloff's published results.[13] His values, adjusted to the same basis as ours, are (for the ω-hydroxyalkanoic acids) $C_{12}=20$ per cent; $C_{14}=50$ per cent; $C_{16}=30$ per cent. Our values are $C_{12}=24$ per cent; $C_{14}=44$ per cent; $C_{16}=32$ per cent; clearly the effect of environment is not important and the ω-hydroxyalkanoic acid pattern appears to be species specific.

The most recent classification of the pines is that due to Critchfield and Little,[7] who have raised a number of Shaw's[5] subspecies to specific rank. The species examined by us in the present work fall into two Sections of Critchfield and Little's classification as shown in Table 3.

[11] T. KARIYONE *et al.*, *J. Pharm. Soc. Japan* **16**, 1 (1962).
[12] J. BOUGAULT and L. BOURDIER, *J. Pharm. Chim.* **29**, 561 (1909); **30**, 10 (1909); **3**, 101 (1911).
[13] E. VON RUDLOFF, *Can. J. Chem.* **37**, 1038 (1959).

TABLE 3. *Gymnospermae. Pinus* SPECIES. (CLASSIFICATION OF CRITCHFIELD AND LITTLE).[7]
THE COMPOSITION OF THE ACIDIC FRACTION FROM THE LEAF WAX ESTERS (EXPRESSED AS MOLE PERCENTAGE OF EACH FRACTION)

Leaf Age	Species	Section	Sub-section		ω-Hydroxy acids					*n*-Fatty acids						
					C_{18}	C_{16}	C_{14}	C_{12}	%	$C_{18:u}$*	C_{18}	$C_{16\ u}$*	C_{16}	C_{14}	C_{12}	%
Old	*P. leiophylla* Schl. and Cham.	Ternatae	Leiophyllae	(1)		19	44	37								
Young	*P. roxburghii* Sarg.	Ternatae	Canariensis	(2a)		22	32	46	84		1		3	18	78	16
Old	*P. roxburghii* Sarg.	Ternatae	Canariensis	(2b)		36	28	36	97		6		22	43	29	3
Old	*P. pinaster* Ait.	Pinus	Sylvestres	(3)		21	7	73	89		13		9	7	71	11
Old	*P. massoniana* Lamb.	Pinus	Sylvestres	(4)		52	41	7	82				19	65	16	18
Old	*P. merkusii* Jungh and de Vries	Pinus	Sylvestres	(5)	8	58	24	10	95·5				23	24	52	4·5
Old	*P. insularis* Endl.	Pinus	Sylvestres	(6)		47	35	17								
Young	*P. engelmannii* Carr.	Pinus	Ponderosae	(7a)		3	50	47	60	36	12	7	28	8	9	40
Old	*P. engelmannii* Carr.	Pinus	Ponderosae	(7b)		17	38	45	91				8	31	61	9
Old	*P. montezumae* Lamb.	Pinus	Ponderosae	(8)		19	51	30	83				4	22	74	17
Old	*P. pseudostrobus* Lindl.	Pinus	Ponderosae	(9)		21	68	11	91				3	30	67	9

* $C_{18\ u}$ and $C_{16\ u}$ = total unsaturated acids of particular chain length.

Species 7, 8 and 9 (Table 3) are of Mexican origin and Nos. 8 and 9 are considered as closely allied botanically: a major C_{14} ω-hydroxyalkanoic acid in each supports this relationship. No. 7 is also placed in the same Subsection Ponderosae but in the older leaves has a dominant C_{12} ω-hydroxy acid. In Subsection Sylvestres the species are all of Eurasian origin. No. 4. (from China) and Nos. 5 and 6 (from Burma and the Philippine Islands) having similar C_{16} ω-hydroxyalkanoic acid dominant patterns; No. 3 (Mediterranean region) however has a dominant C_{12} hydroxy acid while the Indian and Himalayan species, No. 2, has a substantially even distribution pattern of the three ω-hydroxy acids.

TABLE 4. *Pinus engelmannii* LEAF WAX (PLOT 32 MAGUGA). TWO SEPARATE ANALYSES FROM EACH TREE SAMPLED

		ω-Hydroxy acids (mole percentage)		
		C_{12}	C_{14}	C_{16}
A	1	46	33	21
	2	49	31	20
B	1	47	37	16
	2	47	38	15
C	1	47	38	15
	2	49	36	15
D	1	48	39	13
	2	43	38	19
E	1	46	37	17
	2	42	38	20
F	1	43	38	19
	2	46	38	16
G	1	45	39	16
	2	44	40	16
H	1	40	39	20
	2	46	38	16
I	1	41	43	16
	2	42	43	15
J	1	41	42	17
	2	39	42	19
K	1	32	49	19
	2	39	46	15
L	1	31	50	19
	2	35	48	17

The difficulties of defining a species and the variable and subjective weighting that can be given to differences in morphology are illustrated in the revision by Critchfield and Little[7] of Shaw's classification[5] of the genus *Pinus* when a number of variants, considered by Shaw to be of sub-specific rank, were raised to full specific value by the later taxonomists. It seemed to us to be of value to examine the degree of variation (from a chemical viewpoint)

between individual members of a small plantation population in which the members were derived from seed of known origin, and in which the members would, from all taxonomic criteria, be regarded as within a defined species. For the initial examination, duplicate samples of foliage were collected from twelve individual trees of *Pinus engelmannii* growing in Plot 32 at the EAAFRO arboretum, Maguga, near Nairobi. The results of the analyses of the ω-hydroxy acids from the estolide fraction of the leaf wax are given in Table 4, where they are arranged in order of decreasing percentage of the shortest chain component, 12-hydroxydodecanoic acid. It can be seen that duplicate samples from the same tree can show a difference of up to 7 per cent (example K) in the molar percentage of the C_{12}-hydroxy acid, while over the whole series of individual trees the range of variation is between 31 and 49 per cent. For the C_{14}-component the difference is only up to 3 per cent on duplicate samples from the same tree but the overall range is inversely proportional to the C_{12}-component, while the relatively unimportant component, C_{16}, shows only a small variation from one tree to another.

A similar result was obtained from the examination of another plot (Plot 235, Maguga) in which *P. hartwegii* Lindley was under cultivation: again the seed was of known origin and the individual trees would be accepted as being within the definition of a species. Only five trees were sampled, the results of the ω-hydroxy acid analyses being shown in Table 5, arranged in order of descending molar percentage of the major component, C_{14}. Single foliage samples were collected except in the case of tree E, where a duplicate sample was used to confirm the result.

TABLE 5. *P. hartwegii* LEAF WAX (PLOT 235, MAGUGA). INDIVIDUAL TREES SAMPLED

	ω-Hydroxy acids (mole percentage)		
	C_{12}	C_{14}	C_{16}
A	23	65	12
B	24	63	13
C	28	62	10
D	27	61	12
E_1*	32	55	13
E_2*	32	54	14

* Replicate analyses from one tree.

Individual trees may show a variation of up to 11 per cent from one another but nevertheless the range of variation is sufficiently small to distinguish the species clearly from *P. engelmanii*. It is of interest that Harrison (Ref. 3, p. 444) in his summary of the relationships in the *Pinus montezumae* Lambert complex describes *P. hartwegii* as a variety of *P. montezumae*. Comparison of the range of variation in the sampled *P. hartwegii* (Table 5) with an isolated analysis of *P. montezumae* (No. 8 in Table 3) shows that while the relative proportions of the three principal ω-hydroxy acids are similar the *P. montezumae* result falls outside the *P. hartwegii* range. Close relationship between the two is however apparent.

To check further the range of variation in populations, two plots of *P. ayacahuite* Ehrenberg (Plots 239 and 240, Maguga), grown from different seed samples, were examined in the same way. The results are shown in Table 6, where the individual trees are arranged in decreasing percentage of the major component, C_{14}, but irrespective of origin. With a range of variation of 10 per cent in the major component between different specimens, no distinction can be made between the populations of the two plots. Although the major component is the same as for the previously described *P. hartwegii* (Table 5), the median value is significantly different, and a further clear difference between the species is in the relative order of importance of the other two ω-hydroxy acids.

TABLE 6. *Pinus ayacahuite* LEAF WAX (PLOTS 240 AND 239, MAGUGA). ONE ANALYSIS FROM EACH TREE

	ω-Hydroxy acids (mole percentage)		
Plot	C_{12}	C_{14}	C_{16}
239	5	74	21
239	7	72	21
240	4	71	25
240	7	71	22
240	13	71	15
239	9	71	20
240	11	70	19
240	11	65	24
240	12	65	24
239	12	64	24

A final experiment in this series was carried out to compare the two species *P. insularis* Endlicher and *P. kesiya* Royle ex Gordon (formerly *P. khasya* Royle), which are considered by some authorities to be synonymous (cf. Ref. 3, p. 436). Single trees from four plots (Nos. 245 and 246 for *P. insularis* and Nos. 27 and 132 for "*P. khasya*") were sampled: the results of the analyses are shown in Table 7, arranged in decreasing percentage of the major component, C_{16}, irrespective of the plot or species. It can be seen that the range of variation in *P. insularis* is from 57 per cent to 45 per cent of C_{16} and that the two specimens of "*P. khasya*" fall well within this range. Furthermore, the relative abundance of the two minor components, C_{14} and C_{12}, are similar in all the specimens examined. On the basis of this chemical factor, then, there appears to be no discernible difference between the two "species" and the contention that they fall within the same species group can be supported.

Although much further work is required, supported by large numbers of analyses with a full statistical treatment of the variation within populations and the significance of correlations, there seems here to be an initial indication of the possibility of defining a species or a species group by a single chemical characteristic. Such a characteristic could be readily

TABLE 7. *P. insularis* AND "*P. khasya*" LEAF WAXES (CONSIDERED PROBABLY THE SAME SPECIES). SINGLE TREES SAMPLED FROM FOUR PLOTS AT MAGUGA

Plot.		ω-Hydroxy acids (mole percentage)		
		C_{12}	C_{14}	C_{16}
245	*P. insularis*	18	26	57
246	*P. insularis*	19	25	56
246	*P. insularis*	19	29	52
245	*P. insularis*	22	28	50
27	*P. khasya*	16	37	47
246	*P. insularis*	18	35	47
132	*P. khasya*	21	34	45
245	*P. insularis*	23	32	45

determined quantitatively by analysis and might offer an easier approach than the alternative of a metrical analysis of some less easily defined morphological character in a population.

Comparative analyses of the major fraction of the leaf waxes of Cupressaceae species are reported in Table 8. As in the *Pinus* species reported above, the light petroleum insoluble fraction of the leaf wax was found to consist largely of estolides formed from a limited range of ω-hydroxyalkanoic acids. Unless otherwise stated the leaf waxes were extracted from old leaves, since in this series a significant variation in composition of estolide was observed between young and old leaves (cf. Table 8, Nos. 1, 2 and 10).

In contrast to the alkane analyses in Table 1, the ω-hydroxy acid analyses reported in Table 8 can be used to divide the genus *Cupressus* into two distinct groups. *Cupressus funebris* Endlicher (No. 3) and *C. torulosa* Don (No. 2) both show, for old leaves, a dominant 16-hydroxyhexadecanoic acid in the estolide fraction; these two species are from the Old World. All the other species of *Cupressus* in Table 8 are characterized by a dominant C_{12} ω-hydroxy acid in the estolide fraction and are derived from the New World (if it is accepted that *C. lusitanica* is of New World origin). The close similarity of the *C. lusitanica* analysis (No. 5) with those of *C. lindleyi* (No. 7) and *C. benthamii* (No. 6) both for alkanes and ω-hydroxy acids is in keeping with Little's contention[14] that *C. benthamii* should be considered as a sub-species of *C. lusitanica* and with Dyson's reaffirmation (*vide* following paper) that *C. lusitanica* and *C. lindleyi* are synonymous (cf. also Ref. 3, p. 207). Certainly the eighteenth-century botanists' belief that *C. lusitanica* was derived from *C. torulosa* is completely excluded on the evidence of the ω-hydroxy acid analyses.

On the basis of the ω-hydroxyalkanoic acid content of the leaf wax, the genus *Callitris* is clearly differentiated from *Cupressus* by the presence, in significant quantity, of 18-hydroxy octadecanoic acid, as well as the C_{16}, C_{14} and C_{12} members of the homologous series, in all the species examined. Otherwise the *Callitris* ω-hydroxy acid patterns are more akin to the Old World *Cupressus* species in the relative abundance of the C_{16} and C_{12} components.

[14] E. L. LITTLE, *Check List of the Native and Naturalised Trees of the United States*. Handbook 41, Forest Service, U.S. Dept. of Agriculture, Washington, D.C. (1953).

TABLE 8. *Gymnospermae. Cupressaceae* SPECIES. COMPOSITION OF THE ACIDIC FRACTION FROM THE LEAF WAX ESTERS (EXPRESSED AS MOLE PERCENTAGE)

Leaf age	Species		ω-Hydroxy acids					*n*-Fatty acids						
			C_{18}	C_{16}	C_{14}	C_{12}	%	$C_{18:1}$	C_{18}	$C_{16:1}$	C_{16}	C_{14}	C_{12}	%
	Sub-family Cupressoideae Cupressus													
Young	*C. arizonica* Greene	(1a)		6	16	78	92	27	6	3	21	13	29	8
Old		(1b)		19	13	68	95		4		11	17	68	5
Young	*C. torulosa* Don.	(2a)		39	19	42	85	22	5	9	20	35	9	15
Old		(2b)		65	17	18	95		7		17	55	21	5
Old	*C. funebris* Endl.	(3)		60	30	10	95		10		32	48	9	5
Old	*C. macrocarpa* Hartw.	(4)		34	11	55	96				15	10	75	4
Old	*C. lusitanica* Mill.	(5)		19	8	73	94		13		22	12	53	6
Old	*C. benthamii* Endl.	(6)		22	7	71	95		8		20	9	63	5
Old	*C. lindleyi* Klotzsch	(7)		22	11	67	94				25	15	60	6
Old	*C. nevadensis* Abrams	(8)		25	14	61	95				26	10	64	5
Old	*C. cashmeriana* Royle	(9)		63	20	17								
	Sub-family Callitroideae Callitris													
Young	*C. columellaris* F. Muell.	(10a)	10	37	29	24	88	41	9	11	23	11	5	12
Old		(10b)	15	34	42	9	98		17		23	38	22	2
Old	*C. hugelii* (Carr.) Franco	(11)	9	49	31	15	95·5		7		20	23	50	4·5
Old	*C. preissii* Miq.	(12)	11	59	10	20	95		25		35	6	34	5
Old	*C. endlicheri* Comb.	(13)	11	43	31	16	95		27		33	11	29	5
Old	*C. rhomboidea* R. Br.	(14)	26	45	18	11	97		19		16	43	22	3
	Widdringtonia													
Old	*W. juniperoides* Endl.	(15)		25	3	72								
Old	*W. juniperoides* Endl.			27	3	70								
Old	*W. schwarzii* Manl.	(16)		32	9	59								
Old	*W. cupressoides* Endl.	(17)		40	8	52								
Old	*W. cupressoides* Endl.			41	8	51								
Old	*W. whytei* Rendle	(18)		48	4	48								

In contrast to *Callitris*, the genus *Widdringtonia*, which is in the same sub-family Callitroideae, does not contain detectable amounts of the C_{18}-ω-hydroxy acid in its leaf waxes and has very low percentages of the C_{14}-homologue in each of the species examined. This distribution pattern of chain lengths, which is also present to a less marked extent in the New World *Cupressus* species, is unusual among leaf wax constituent homologous series so far examined. The earlier work of Bougault and Bourdier,[12] in which the C_{16} and C_{12} ω-hydroxy acids were isolated from *Juniperus sabina* L., but not the C_{14} homologue, was no doubt controlled by a similar distribution pattern in this species, which we have not, however, re-examined.

In conclusion it can be stated that, for those Gymnosperms investigated in this work, the major leaf wax constituents, the ω-hydroxyalkanoic acids, appear to form valuable chemotaxonomic criteria both in the separation of genera and in the identification of species and of species groups within genera. On the other hand, alkanes, which form only a minor proportion of the leaf waxes, do not afford a useful guide in the genera *Cupressus*, *Juniperus*, *Callitris* and *Widdringtonia* of the Cupressaceae and in the genus *Pinus* (Pinaceae), although the work of Eglinton *et al.*[9] on other families of conifers has been more rewarding. A more detailed study of the ω-hydroxy acids as chemotaxonomic criteria in the genus *Pinus* is in progress.

EXPERIMENTAL

Methods for the isolation of the leaf cuticular waxes and for the analysis of the alkane fraction are as previously described.[8]

Isolation and Analysis of the Alkanoic and ω-Hydroxyalkanoic Acids

Leaf cuticular wax (50–100 mg) was added to 25 ml of 10% KOH in rectified spirit and heated under reflux for 4 hr. The solution was then evaporated almost to dryness, diluted to 25 ml with distilled water and heated to boiling. After cooling somewhat, three extractions with hot light petroleum (b.p. 80–100°) were made to remove the non-saponifiable material. The aqueous solution after acidification was extracted with ether to obtain the alkanoic and hydroxy-alkanoic acids which were immediately methylated with diazomethane and then acetylated in the cold using the method of Fritz and Schenk.[15] The ethereal solution of methyl esters and methyl ester acetates was chromatographed on an alumina column (Peter Spence Type H,100/200 mesh, 10 cm × 0·8 cm), packed dry and eluted with ether. The mixed esters were recovered by evaporation of the ether eluate.

The i.r. absorption spectrum of the mixed esters in CCl_4 solution showed strong maxima at 2950, 1750 and 1240 cm^{-1} and closely resembled the spectrum of an authentic sample of methyl 15-acetyloxypentadecanoate.

The mixed esters were analysed by gas chromatography under standard conditions (Pye Argon chromatograph, 90 cm column packed with 10% polyethyleneglycol adipate on Celite, carrier gas flow 125 ml/min, temperature 160°). Alkanoic acid chain lengths were determined by use of a standard mixture of oleic, stearic, palmitic, myristic and lauric acid methyl esters.

Verification of the hydroxy acid carbon numbers and the primary nature of the hydroxyl group was established as follows:

(*a*) Wax acetyloxyesters and methyl 15-acetyloxypentadecanoate admixed gave a straight line plot of the logarithm of the retention time against carbon number,

(*b*) Chromic acid oxidation of the wax hydroxy acids to dioic acids of identical chain length was shown by co-gas chromatography of their methyl esters with authentic samples of dimethyl decane-1,10-dioate, dimethyl dodecane-1,12-dioate and dimethyl pentadecane-1,15-dioate,

(*c*) Reduction of the wax hydroxy acid methyl esters with lithium aluminium hydride to the corresponding α,ω-diols and gas chromatography of the acetates of the latter established homology and admixture with authentic samples of the diacetates of the C_{10}, C_{12} and C_{15}-diols proved identity.

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[15] J. S. FRITZ and G. H. SCHENK, *Anal. Chem.* **31**, 1808 (1959).