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STUDIES ON PLANT CUTICULAR WAXES—V.

THE WAX COATINGS OF PINE NEEDLES: A TAXONOMIC SURVEY

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Abstract—A gas-chromatographic study has been made of the ω-hydroxy acid composition of the leaf wax estolide fractions of thirty-nine *Pinus* species and the results compared with three major taxonomic arrangements of the genus. Although the ω-hydroxy acid compositions do not allow closely related species to be distinguished from one another, broad areas of agreement between the botanical classifications and the leaf wax estolide compositions are evident.

INTRODUCTION

A NUMBER of workers have assessed chemical constituents of *Pinus* species for their potential taxonomic value. Notable contributions to this field are the painstaking investigations of phenolic heartwood constituents by Erdtman,[1] leaf wax estolides and biflavonyls by Kariyone *et al.*,[2] and steam volatile components of pine oleoresins by Mirov.[3]

In Part III[4] of the present series ω-hydroxy acid compositions of leaf wax estolide fractions of several *Pinus* species were reported. Variation in composition with age was noted for *Pinus engelmannii* and *P. roxburghii* and intraspecific variation among members of small plantation populations was established for *P. engelmannii, P. hartwegii, P. ayacahuite, P. insularis* and *P. khasya*, when it was concluded that the results of a more extensive examination of the genus might prove to be taxonomically significant.

This paper subjects three important taxonomic arrangements of the genus to a process of evaluation, on the basis of relationships within the group, by comparing the classifications of Shaw,[5] Pilger[6] and Critchfield and Little, Jr.[7] with *Pinus* species leaf wax ω-hydroxy acid compositions.

RESULTS AND DISCUSSION

Analysis of the leaf wax from a particular tree poses a difficulty in that its composition might represent an extreme of intraspecific variation within a small population. To avoid

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[1] H. ERDTMAN, in *Chemical Plant Taxonomy* (edited by T. SWAIN), Academic Press, London and New York (1963).

[2] T. KARIYONE, M. TAKAHASHI, K. WATANABE, H. AGETA, H. ISOI, H. Y. HSU, N. KAWANO, T. SAWADA and Y. FUKRI, *J. Pharm. Soc. Japan* **16**, 1 (1962).

[3] N. T. MIROV, *Composition of Gum Terpentines of Pines.* U.S. Dept. Agr. Tech. Bull. No.1239, Washington, D.C. (1961).

[4] G. A. HERBIN and P. A. ROBINS, *Phytochem.*, **7**, 1325 (1968).

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

[6] R. PILGER, in *Die Naturlichen Pflanzenfamilien*, Vol. 13, pp. 332–341 (edited by P. ENGLER and K. PRANTL), Duncker and Humbolt, Berlin (1960).

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

this possibility and to minimize age-dependent variation, old, fading needles were removed from ten trees of each species, when this number was available, and pooled prior to wax extraction. It seemed to us that an analysis of combined samples from several trees would be more representative of that species, offering a suitable basis for comparison with populations widely separated geographically. Triplicate analyses of each of five species are shown in Table 1 illustrating the reproducibility obtained.

The pines comprise a genus clearly defined from other conifers. The number of postulated species has increased with successive revisions of the genus. Three major classifications are those of Shaw,[5] sixty-four species; Pilger,[6] seventy-eight species; and Critchfield and Little, Jr.,[7] ninety-four species. Set out in Table 2 are the ω-hydroxy acid compositions of thirty-nine species classified according to Critchfield and Little, Jr. All analyses reported against plot number are of waxes from pines of established seed origin growing under small-scale plantation conditions at the East African Agriculture and Forestry Research Organisation (E.A.A.F.R.O.), Muguga, near Nairobi. These plots are in close proximity to one another, thus ensuring that any non-genetic interspecific variation in leaf estolide composition would be minimal. Waxes from eleven species, ten of which were not available at Muguga, were obtained from Sweden. In order to ascertain the significance of environmental influences on wax composition, a third sample of *P. ponderosa* leaf wax, collected from trees native to the Black Hills of South Dakota, U.S.A., was analysed for comparison with the samples of *P. ponderosa* leaf waxes from Sweden and Muguga. In Table 2, waxes originating from

TABLE 1. TRIPLICATE ANALYSES OF THE ω-HYDROXY ACIDS FROM PINE NEEDLES OF FIVE *Pinus* SPECIES

Pinus species		Mole percentage		
		C_{12}	C_{14}	C_{16}
P. insularis	(1)	21	22	57
	(2)	18	26	56
	(3)	21	30	49
P. ayacahuite	(1)	12	70	18
	(2)	9	68	23
	(3)	8	65	27
P. caribaea	(1)	66	16	18
	(2)	64	18	18
	(3)	62	19	19
P. cubensis	(1)	53	15	32
	(2)	50	15	35
	(3)	49	14	37
P. pseudostrobus	(1)	13	66	21
	(2)	11	60	29
	(3)	11	54	35

Needles from several trees of each species were pooled prior to analysis. ω-Hydroxy acids are designated by their carbon chain length.

TABLE 2. THE ω-HYDROXY ACIDS PRESENT IN THE ESTOLIDE FRACTION OF PINE NEEDLE WAXES. (CLASSIFICATION ACCORDING TO CRITCHFIELD AND LITTLE, JR.)

Section	Subsection	*Pinus* species	Plot number	Mole percentage (C=carbon chain length)		
				C_{12}	C_{14}	C_{16}
Subgenus *Strobus*						
Strobus	*Cembrae*	1. *koraiensis* Sieb and Zucc.	Sweden	15	52	33
		2. *cembra* L.	Sweden	23	50	27
	Strobi	3. *armandii* Franch.	Sweden	28	32	40
		4. *peuce* Griseb.	Sweden	12	64	24
		5. *strobus* L.	241	9	57	34
		6. *ayacahuite* Ehrenb.	239	9	68	23
Subgenus *Pinus*						
Ternatae	*Leiophyllae*	7. *leiophylla* Schiede and Deppe	31	35	45	20
	Canarienses	8. *roxburghii* Sarg.	19	27	38	35
		9. *canariensis* C. Smith	18	42	43	15
	Pineae	10. *pinea* L.	93	23	59	18
Pinus	*Sylvestres*	11. *massoniana* Lamb.	103	8	28	64
		12. *merkusii* Jungh. and de Vriese	107	12	26	62
		13. *insularis* Endl.	246	18	26	56
		14. *yunnanensis* Franch.	Sweden	53	13	34
		15. *mugo Turra*	Sweden	35	25	40
		16. *sylvestris* L.	Sweden	42	17	41
		17. *nigra* Arnold	Sweden	53	28	19
		18. *halepensis* Mill.	26	40	32	28
		19. *pinaster* Ait.	214	73	7	20
	Australes	20. *occidentalis* Sw.	28	65	22	13
		21. *cubensis* Gris.	242	50	15	35
		22. *caribaea* Morelet	243	64	18	18
		23. *elliottii* Engelm.	106	54	21	25
		24. *taeda* L.	33	53	18	29
		25. *echinata* Mill.	34	50	14	36
	Ponderosae	26. *ponderosa* Laws.	133	58	31	11
		26a. *ponderosa* Laws.	U.S.A.	54	31	15
		26b. *ponderosa* Laws.	Sweden	76	24	—
		27. *engelmannii* Carr.	32	46	37	17
		28. *jeffreyi* Grev. and Balf.	Sweden	63	30	7
		29. *montezumae* Lamb.	234	28	60	12
		30. *hartwegii* Lindl.	235	29	55	16
		31. *pseudostrobus* Lindl.	228	11	60	29
		32. *douglasiana* Mart.	232	19	56	25
	Sabinianae	33. *sabiniana* Dougl.	29	14	61	25
	Contortae	34. *banksiana* Lamb.	Sweden	87	13	—
		35. *virginiana* Mill.	35	61	23	16
		36. *clausa* Vasey	36	44	19	37
	Oocarpae	37. *oocarpa* Schiede	219	34	39	27
		38. *patula* Schiede and Deppe	227	30	39	31
		39. *radiata* Don.	15	20	37	43

Sweden and the U.S.A. are designated "Sweden" and "U.S.A." respectively under the column headed "plot number".

Inspection of three *P. ponderosa* analyses, Table 2, numbers 26, 26a and 26b, shows close agreement between the compositions of the U.S.A. and Muguga waxes. The wax from Sweden contains a noticeably higher proportion of 12-hydroxydodecanoic acid. However, all three estolide fractions are clearly dominated by the C_{12}-homologue.

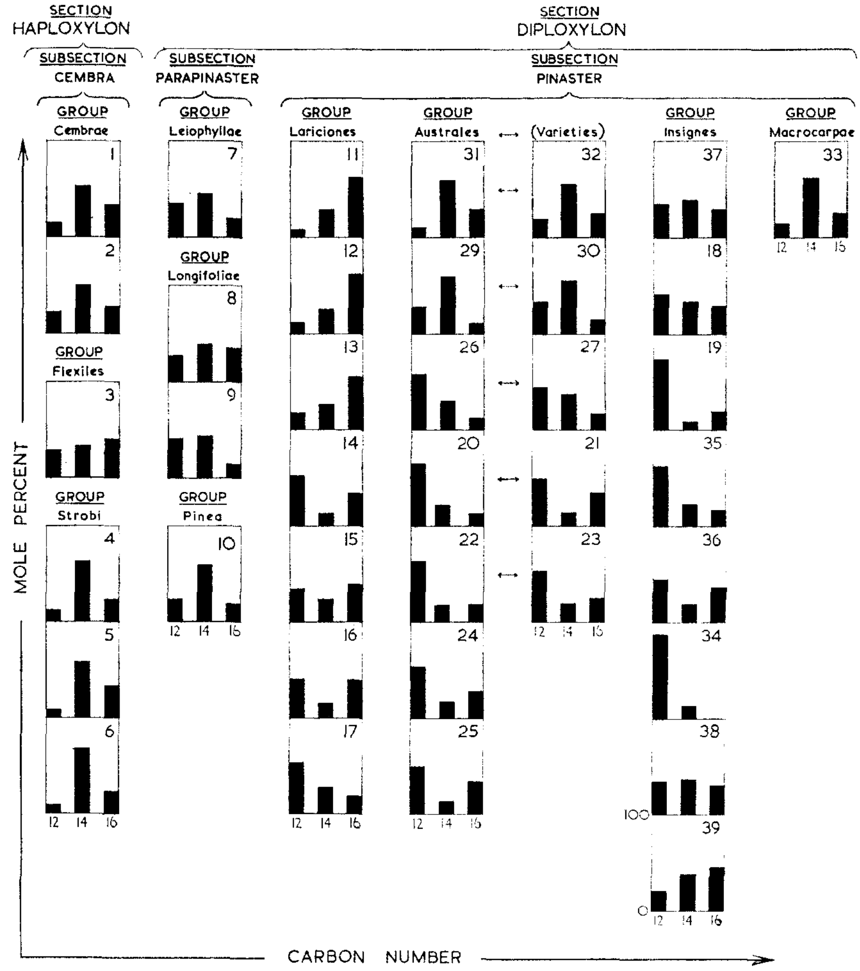

FIG. 1. DISTRIBUTION, IN MOLE PERCENTAGE, OF THE ω-HYDROXY ACIDS OF THE SURFACE WAX ESTOLIDE FRACTION OF INDIVIDUAL *Pinus* SPECIES ARRANGED ACCORDING TO THE CLASSIFICATION OF SHAW.[5]

Histogram numbers refer to the positions of species in Table 2. ω-Hydroxy acids are designated by carbon chain length.

To facilitate the following discussion, each estolide ω-hydroxy acid composition is represented in the form of a histogram which is numbered according to the position of the species in Table 2. In Figs. 1, 2 and 3, histograms are arranged as per the classifications of Shaw, Pilger and Critchfield and Little, Jr., respectively.

For a phylogenetic arrangement of the genus, Shaw chose certain characteristics of the seed and cone, arranging them in an order which, he considered, reflected progressive evolutionary change. Shaw divided his sixty-four species into two sections, namely, *Haploxyl-*

on and *Diploxylon*. He considered *Haploxylon* species to be more primitive than *Diploxylon* species.

Referring to Fig. 1, six *Haploxylon* species presently studied, numbers 1–6 inclusive, show 14-hydroxytetradecanoic acid (C_{14}) as the major acid, with one exception, *P. armandii*, 3, which has 16-hydroxyhexadecanoic acid (C_{16}) as its major constituent. 12-Hydroxydodecanoic acid (C_{12}) is the minor constituent of all six species. Apart from *P. armandii*, 3, whose pattern is sufficiently dissimilar to warrant inclusion in a separate group from the other *Haploxylon* species listed, ω-hydroxy acid patterns 1, 2, 4, 5 and 6 resemble each other so closely that they appear to be of little value in either delineating species or distinguishing members of group *Cembrae* from those of group *Strobi.*

In section *Diploxylon*, species of sub-section *Parapinaster*, as Shaw points out, have "... essential characters of *Diploxylon* with important characters of *Haploxylon*". It is interesting that all four species examined in this sub-section, i.e. *P. leiophylla*, 7, *P. roxburghii*, 8 (Shaw refers to this species as *P. longifolia* Roxburgh), *P. canariensis*, 9, and *P. pinea*, 10, also contain C_{14} as the major acid, apparently a common feature of *Haploxylon* species. However, within this sub-section, C_{12} has displaced C_{16} from its position as the second most abundant acid in the leaf estolide fraction.

In sub-section *Pinaster*, Shaw has four groups arranged according to their hypothetical progressive characters, but he hastens to add that species in this sub-section are very difficult, if not impossible, to classify by the usual morphological method, which groups all species under a few characters assumed to be invariable and of fundamental importance. Such a method can be successfully applied to the Soft Pines (*Haploxylon*) and to some of the Hard Pines (*Diploxylon*), but cannot be applied to all Hard Pines without forcing some of them into unnatural associations. Therefore, some rearrangement of Shaw's four groups in this sub-section is anticipated.

Seven species, native to the Old World, are listed in the first of these four groups, namely, *Lariciones*. *P. massoniana*, 11, *P. merkusii*, 12, and *P. insularis*, 13, are of Asian origin, exhibiting patterns almost indistinguishable from one another and characterized by a high C_{16} content. *P. sylvestris*, 16, is the most widely distributed of all pines ranging throughout northern Eurasia from Scotland and as far south as southern Spain and northern Greece and Turkey. In Europe it is associated with *P. mugo*, 15 (*P. montana* Miller according to Shaw), where hybrids of the two species are believed to occur, see Fitschen[8] (1930), p. 430. Both species have similar patterns in that they contain approximately equal proportions of C_{12} and C_{16} with C_{14} as the minor component. The fourth Asian pine, *P. yunnanensis*, 14 (Shaw's *P. sinensis* Lamb.), has a higher C_{12} content but C_{14} remains the minor constituent. Wax from *P. nigra*, 17, contains more than 50 per cent of C_{12} and less than 20 per cent of C_{16}. In this latter respect it has less in common with its associates in the group. Also noteworthy is the displacement of C_{14}, the most abundant acid of the preceding groups, from the major position by either its higher or lower homologue.

Species in group *Australes* are found in the New World. Numbers 32, 30, 27, 21 and 23 were considered varieties by Shaw. *P. douglasiana*, 32, a variety of *P. pseudostrobus*, 31; *P. hartwegii*, 30, a variety of *P. montezumae*, 29; *P. engelmannii*, 27, a variety of *P. ponderosa*, 26; *P. cubensis*, 21, a variety of *P. occidentalis*, 20, and *P. elliottii*, 23, a variety of *P. caribaea*, 22. Comparison of a species with its postulated variety shows close resemblance between the two patterns, the hydroxy acids occurring in the same order of magnitude in both patterns, with the exception of 20 and 21, the former containing C_{16} and the latter C_{14} as the minor

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[8] J. FITSCHEN, *Handbuch der Nadelholzkunde*, 3rd edition, Paul Parey, Berlin (1930).

constituent. Shaw's seven full species fall into two clearly delimited groups. *P. pseudostrobus*, 31, and *P. montezumae*, 29, natives of Mexico, have C_{14}-dominant patterns distinct from the major C_{12} patterns of the remaining five species.

Within the group *Insignes* is found the greatest diversification in pattern profiles. These species are largely found in the New World but *P. halepensis*, 18, and *P. pinaster*, 19, originate from Europe. Three New World pines from eastern North America, *P. banksiana*, 34, *P. virginiana*, 35, and *P. clausa*, 36, and two European pines, 19 and 18, show C_{12} as the major acid. Estolide fractions of *P. oocarpa*, 37, and *P. patula*, 38, waxes narrowly contain C_{14} as their major acid and the western North America pine, *P. radiata*, 39, is unusual in this group in respect of its leaf wax containing C_{16} in highest concentration. It also has the distinction of being the only New World species, which we have analysed, to exhibit a major C_{16} pattern.

The lone species investigated from group *Macrocarpae*, namely, *P. sabiniana*, 33, with its major C_{14} pattern appears to be chemically related to the Central America pines.

From what has been said above it can be concluded that, in general, species Shaw considered to be most primitive, i.e. those from section *Haploxylon* and sub-section *Parapinaster* of section *Diploxylon*, are characterized by the presence of C_{14} as the major estolide acid. Similarly, species considered relatively more advanced, e.g. those of group *Lariciones*, appear to be characterised by the dominance of C_{16}, and species Shaw considered to be most advanced contain C_{12} as their major leaf wax ω-hydroxy acid. This curious progression whereby C_{14} is replaced by C_{16} followed, as it were, by a regression to a C_{12}-dominant estolide composition may have important biosynthetic implications which for the present remain obscure. It is noteworthy that none of the leaf wax estolide fractions from nine *Cupressus* and four *Widdringtonia* species, reported in Part III[4] of this series, exhibited a C_{14}-dominant pattern and only one of five *Callitris* species examined, namely, *C. coulumellaris*, showed this characteristic. The almost total eclipse of C_{14} is particularly evident in the four *Widdringtonia* species.

Pilger relied mainly on needle numbers and the position of the resin canals for his classification. He differs slightly from Shaw in his treatment of *Haploxylon* species, but important differences are manifest in their treatment of *Diploxylon* species. Moreover, it is of interest to point out now that, when the ω-hydroxy acid distribution patterns are arranged according to Pilger, a graded group relationship is less readily discernible.

Turning to Fig. 2, in Pilger's sub-genus *Diploxylon*, two species studied of section *Sula*, i.e. *P. roxburghii*, 8 (Pilger lists this species as *P. longifolia*), and *P. canariensis*, 9, show substantially different patterns, but Shaw has also grouped them together (see Fig. 1, group *Longifoliae*). Six species from section *Eupitys: P. massoniana*, 11, *P. merkusii*, 12, *P. yunnanensis*, 14, *P. mugo*, 15, *P. sylvestris*, 16, and *P. nigra*, 17, are common to Shaw's group *Lariciones* (Fig. 1). The seventh member of Shaw's group, an Asian pine, *P. insularis*, 13, is separated from two other Asian species, 11 and 12, and placed by Pilger in a later section *Khasia*. The chemical evidence does not support this change, all three species clearly possessing C_{16}-dominant patterns.

Although species of section *Banksia* exhibit a range of patterns, C_{12} is invariably the major acid present in agreement with their inclusion in the same section. The lone C_{14}-dominant *P. pinea*, 10, occurs in section *Pinea* which, however, is further removed from the similarly endowed *Haploxylon* species than Shaw's group *Pinea* (Fig. 1).

There appears to be little connexion between Shaw's group *Australes* and Pilger's section *Australes*. Two C_{12}-dominant pines, namely, *P. occidentalis*, 20, and *P. caribaea*, 22, are included in both groupings. The third pine of section *Australes* (Fig. 2), *P. occarpa*, 37, shows a more even distribution of its constituent ω-hydroxy acids.

Species of section *Pseudostrobus* are, on a chemical basis, readily divisible into two groupings. *P. leiophylla*, 7, *P. montezumae*, 29, and *P. pseudostrobus*, 31, show a close relationship, the leaf waxes of all three possessing a high C_{14} content. Estolide fractions of *P. ponderosa*, 26, *P. engelmannii*, 27, and *P. jeffreyii*, 28, are dominated by C_{12}. Least agreement between the chemical characters of species and their taxonomic grouping occurs in section *Taeda* in that *P. taeda*, 24, *P. sabiniana*, 33, *P. patula*, 38, and *P. radiata*, 39, exhibit C_{12}-dominant, C_{14}-dominant, C_{14}-major and C_{16}-major patterns respectively.

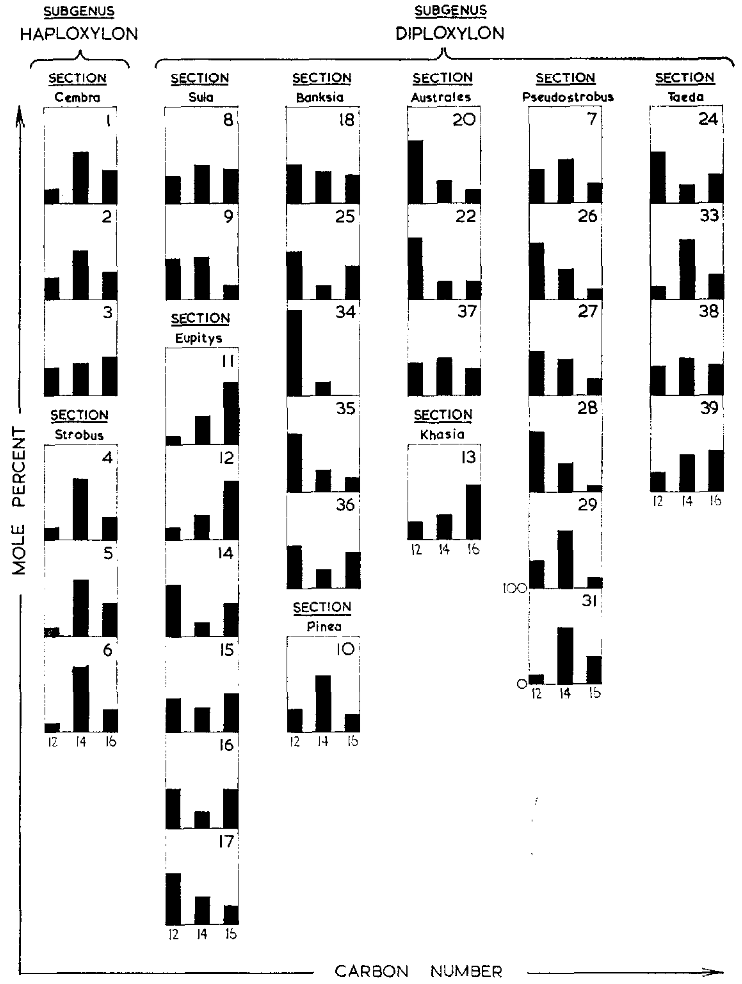

FIG. 2. DISTRIBUTION, IN MOLE PERCENTAGE, OF THE ω-HYDROXY ACIDS OF THE SURFACE WAX ESTOLIDE FRACTION OF INDIVIDUAL *Pinus* SPECIES ARRANGED ACCORDING TO THE CLASSIFICATION OF PILGER.[6]

Histogram numbers refer to the positions of species in Table 2. ω-Hydroxy acids are designated by carbon chain length.

Critchfield and Little, Jr., divided their ninety-four species into three sub-genera: *Ducampopinus*, *Strobus* and *Pinus*. Sub-genus *Ducampopinus* contains one species, which was not available for analysis, while sub-genus *Strobus* contains thirty-one species. Both sub-genera are included in the sub-division *Haploxylon* of Shaw and Pilger. Sub-genus *Pinus* (*Diploxylon* according to Shaw and Pilger) is divided into the two sections *Ternatae* and *Pinus*. Sections *Ternatae* and *Pinus* are equivalent to Shaw's sub-sections *Parapinaster* and *Pinaster* respectively, but bear little relationship to the sections of Pilger's classification Section *Ternatae* is divided into three sub-sections (Shaw's groups). Section *Pinus* is further divided into six sub-sections as against Shaw's four groups. The sub-division of section *Pinus* owes much to Duffield's[9] relationship scheme, which is based on species hybridization experiments, except that Shaw's group *Macrocarpae* has been retained as sub-section *Sabinianae*.

A comparison of Fig. 1 with Fig. 3 shows that Critchfield and Little, Jr., differ largely from Shaw in that they have raised many of Shaw's varieties to full specific rank and have also revised his sub-section *Pinaster* in their section *Pinus*. Their system differs slightly from Shaw's groupings preceding the latter's group *Australes*, apart from the inclusion of *P. halepensis*, 18, and *P. pinaster*, 19, in sub-section *Sylvestres* (Fig. 3), a move which does not invalidate the previous observation made with regard to the species of Shaw's group *Lariciones* that no C_{14}-major species is included in the grouping. The present discussion is, therefore, restricted to Critchfield and Little, Jr's, arrangement of sub-section *Australes* and the subsequent sub-sections.

All species of sub-section *Australes*, 20–25 inclusive, exhibit C_{12}-dominant distribution patterns in accord with the botanical classification—an apparent improvement on Shaw's group *Australes* (Fig. 1). This finding is also in harmony with the results of breeding experiments by Duffield[9] who showed that eastern and southern North America and Caribbean pines appear to form a coherent group.

Seven species included in sub-section *Ponderosae* fall comfortably into two groupings. *P. ponderosa*, 26, and its close associates, *P. engelmannii*, 27, and *P. jeffreyii*, 28, exhibit C_{12}-major distribution patterns whereas those of *P. montezumae*, 29, *P. hartwegii*, 30, *P. pseudostrobus*, 31, and *P. douglasiana*, 32, are C_{14}-dominant. *P. sabiniana*, 33, of sub-section *Sabinianae*, also exhibits this type of distribution pattern.

Species from the last two sub-sections *Contortae* and *Oocarpae* have patterns which broadly agree with their inclusion in their respective sub-sections. Species of sub-section *Contortae: P. banksiana*, 34, *P. virginiana*, 35, and *P. clausa*, 36, have C_{12}-major patterns while the *Oocarpae* species, namely, *P. oocarpa*, 37, *P. patula*, 38, and *P. radiata*, 39, have patterns with a more even distribution of the three ω-hydroxy acids.

While it is unlikely that such a large genus as *Pinus* can be arranged meaningfully on the basis of a few chemical characters, we believe the following observations are pertinent to any contemplated phylogenetic arrangement of the species of this genus. Nine eastern North America and Caribbean pines of section *Pinus*, comprising all species analysed in Critchfield and Little, Jr's, sub-sections *Australes* and *Contortae* (Fig. 3), show, without exception, C_{12} as their major ω-hydroxy acid. *P. clausa*, 36, is the only member whose C_{12} content falls below 50 per cent of the total leaf estolide ω-hydroxy acids. Eight species: 6, 7, 29, 30, 31, 32, 37 and 38, found in Mexico and/or Central America, exhibit C_{14}-major patterns typical of the Soft Pines. Interestingly C_{16} is most frequently the major acid of

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[9] J. W. DUFFIELD, "Relationships and species hybridization in the genus *Pinus*", *Zeitschr. Forstgenetik Forstpflanzenzücht.* **1**, 93 (1952).

pines of Asian origin, cf. 3, 8, 11, 12, 13 and 14. On the contrary, the European Hardwood Pines of sub-genus *Pinus* included in our survey: 10, 15, 16, 17, 18 and 19, show little conformity exhibiting the full range of pattern profiles.

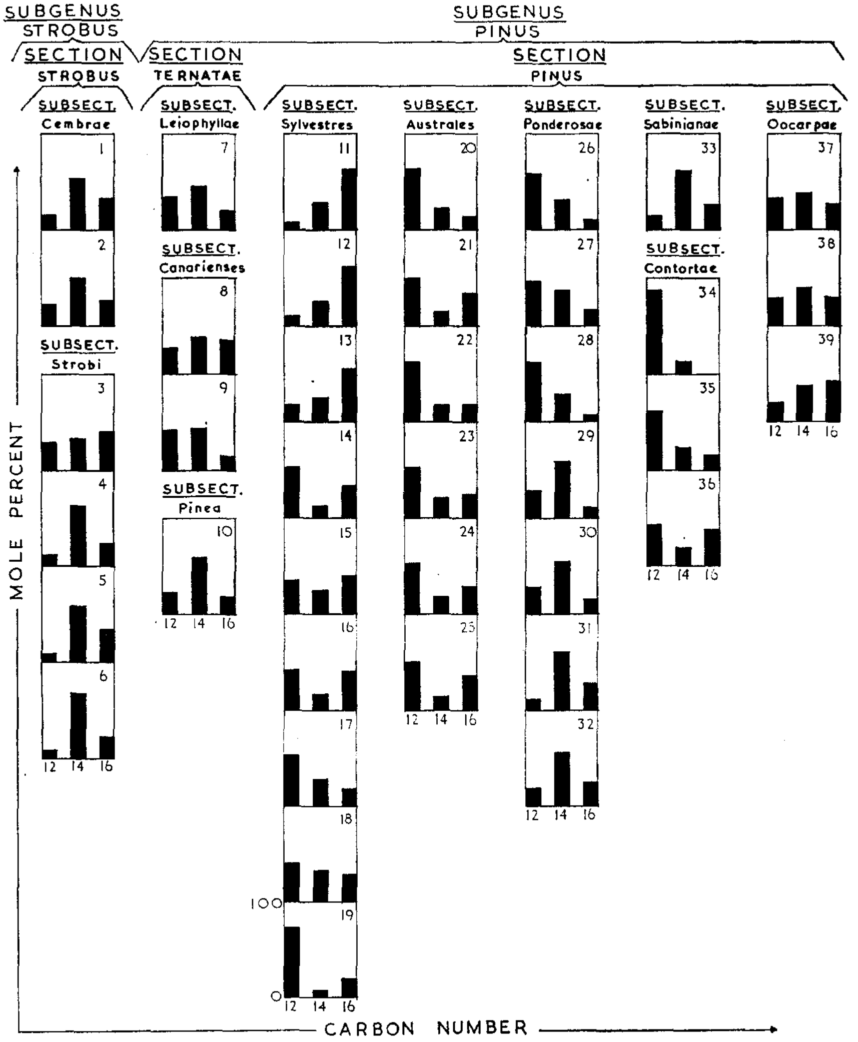

FIG. 3. DISTRIBUTION, IN MOLE PERCENTAGE, OF THE ω-HYDROXY ACIDS OF THE SURFACE WAX ESTOLIDE FRACTION OF INDIVIDUAL *Pinus* SPECIES ARRANGED ACCORDING TO THE CLASSIFICATION OF CRITCHFIELD AND LITTLE, JR.[7]

Histogram numbers refer to the positions of species in Table 2. ω-Hydroxy acids are designated by carbon chain length.

EXPERIMENTAL

Full details of the methods for the extraction of leaf cuticular waxes[10] and for the isolation and analysis of the ω-hydroxyalkanoic acids[4] are as previously described.

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

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