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STUDIES ON PLANT CUTICULAR WAXES—IV.

LEAF WAX ALKANES AS A TAXONOMIC DISCRIMINANT FOR CYPRESSES GROWN IN KENYA

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Abstract—Statistical analyses of the results of gas chromatographic studies of the alkane components of *Cupressus* leaf waxes show that individual trees and species have highly characteristic minor alkane patterns. Species may be diagnosed by such analyses and hybrids have alkane patterns intermediate between the parent species. *Cupressus lusitanica*, which is of disputed native origin, has alkane components characteristic of a New World species.

INTRODUCTION

Cypresses of the genus *Cupressus* L. are not native to East Africa, but eleven species have been introduced to Kenya as ornamental trees, or for timber production. Of these, two, *C. lusitanica* Miller and *C. macrocarpa* Hartw., have proved outstandingly successful timber producers, and have been very extensively planted. They are of major economic importance in the Kenya timber industry.[1,2]

In cultivation, the cypresses present a bewildering range of form and some Kenya crops contain many trees which appear intermediate between known species. Natural hybridization has been postulated as the origin of these intermediate trees.[3,4] A preferred strain of *C. lusitanica* has been selected from this confusing range of types, which shows superior growth and yield for timber production,[5] but its precise origin was puzzling. Dyson[6] made a detailed taxonomic and breeding study of Kenya cypress crops, and the gas chromatographic study of leaf waxes described here was used to confirm and clarify the conclusions of the morphological study.

Taxonomy of the Genus Cupressus *L.*

The genus *Cupressus* comprises a group of evergreen coniferous trees with a circum-polar distribution between latitudes 15 and 45° N. The formal taxonomy of the group is confusing. Individual trees within a species are extremely variable and some species have varieties which differ in appearance more from the typical species than they do from varieties of other species.

[1] S. H. Wimbush, *The Management of Cypress Plantations in Kenya.* Kenya Forest Department Pamphlet No. 11 Nairobi (1945).
[2] J. P. W. Logie, *Annual Report of the Kenya Forest Department for* 1965. Government Printers. Nairobi (1967).
[3] R. M. Graham, *The Cypresses.* Plantation Management Order No. 6, Kenya Forest Department, Nairobi (1949).
[4] A. L. Griffith, *Empire Forest Rev.* **32**, 361 (1953).
[5] W. G. Dyson, *Comm. For. Rev.* **43**, 213 (1964).
[6] W. G. Dyson, Thesis for Doctor of Philosophy submitted to the University of East Africa (1967).

The identification of single specimens, especially in the herbarium, is difficult and botanical opinions differ on the number of species into which the group should be divided. Thus, Wolf[7] recognizes fourteen species occurring in the south-western United States but Little[8] reduces these to only six. Taking a broad concept of species, we may say that there are twelve species in all; five in the Old World and seven in the New World.

C. lusitanica, the species most widely grown in Kenya, is of special interest. It was originally described from Britain, in 1768, from trees introduced from Portugal, but where it was known not to be native. Eighteenth-century botanists believed that it had been introduced from Goa but, as early as 1839, Forbes suspected that it was identical with two Mexican cypresses, later known as *C. lindleyi* Klotsch and *C. benthamii* Endl. This view is now accepted by most botanists (Franco[9]), but in Mexico Martinez' opinion is followed. He maintained that *C. lindleyi*, *C. benthamii* and *C. lusitanica* are three distinct species; the two former being native to Mexico and the latter of unknown (presumably Asian) origin. In Kenya, crops of all three strains were available and, in order to avoid confusion in this paper, will be called by their full specific names.

Experimental Design

To use leaf wax analysis to confirm the morphological study of the origin and identity of the Kenya strain of *C. lusitanica*, we sought to establish three principles:

(i) That *Cupressus* species could be distinguished by comparisons of the alkane fractions of their leaf waxes.
(ii) That Old and New World species could be distinguished.
(iii) That hybrids, having vegetative characters intermediate between the parent species, also have intermediate leaf waxes.

Preliminary work has shown variation with age of conifer wax estolides[10] and of the leaf wax alkanes of *Solandra grandiflora*[11] underlining a need for uniform sampling of leaf material.

Variation between trees of the same *Pinus* species, not attributable to age difference of the leaves examined, has also been observed.[10] Such variations require replication of sampling and statistical treatment of the analytical data.

Although Herbin and Robins[10] were able to separate New World from Old World cypress species by comparing the ω-hydroxy alkanoic acid composition of their leaf wax estolides, which comprise the bulk of the wax, they were unable to distinguish between species on the basis of a comparison of the major leaf wax alkanes, namely C_{33} and C_{35}. It seemed possible that the "fine structure" of leaf alkane patterns, i.e. the relative proportions of minor alkane constituents, might be more discriminating from a taxonomic viewpoint.

Preliminary *Cupressus* leaf alkane analyses were sufficiently encouraging to warrant a more extensive investigation; accordingly the following sampling technique was adopted.

Paired samples from seven individuals of each species were analysed by gas–liquid chromatography for their minor alkane constituents. Eight species, three Old World, four New World and *C. lusitanica* (of disputed origin) were investigated.

[7] C. B. WOLF and W. WAGENER, *El Aliso*, The New World Cypresses, **1**, 1 (1948).
[8] E. L. LITTLE, *Check List of Native and Naturalised Trees of the United States*. Agr. Handb. 41, U.S. Department of Agriculture, Washington, D.C. (1953).
[9] A. FRANCO, *Rev. Agros. Lisboa* **28**, 1 (1945).
[10] G. A. HERBIN and P. A. ROBINS, *Phytochem.* (preceding paper).
[11] G. A. HERBIN, Thesis for Doctor of Philosophy, University of London (1967).

RESULTS AND DISCUSSION

Inspection of representative chromatograms of the alkane fraction of leaf waxes from three species of *Cupressus* confirms that differences between species occur in the relative proportions of the minor alkanes present. Thus the Old World species all show comparatively even proportions of C_{27}, C_{26} and C_{25} alkanes, represented by three adjacent even-sized peaks on the chromatogram. *C. arizonica* and *C. macrocarpa* show relatively large C_{27} and C_{29} peaks respectively. Such proportional differences are readily assessed as ratios. The peak areas were measured by cutting out and weighing and tabulated by species and alkane carbon number. Preliminary inspection of the tabulated data suggested that four ratios: $(C_{25}+C_{26})/C_{27}$; C_{29}/C_{27}; C_{31}/C_{29} and C_{35}/C_{31} would be the most critical. The ratio data calculated for paired samples were subjected to statistical analysis with the following results.

Discrimination of Species

The variance estimates calculated for seven paired samples from each of eight species are presented in Table 1.

TABLE 1.

Source of variation	Degrees of freedom	Variance estimates and variance ratios for stated alkane ratios							
		$\frac{C_{25}+C_{26}}{C_{27}}$		$\frac{C_{29}}{C_{27}}$		$\frac{C_{31}}{C_{29}}$		$\frac{C_{35}}{C_{31}}$	
Between species	7	4·35	18·0***	25·28	13·3***	48·07	25·3***	137·16	28·2***
Between trees within species	48	0·24	0·28 NS	1·90	2·1**	1·90	2·5**	4·86	7·5**
Within trees	56	0·87		0·90		0·75		0·65	

The small variance estimates obtained for samples within individual trees give considerable confidence in the field sampling and laboratory techniques used. Three out of the four sets of ratio data calculated could have been used to discriminate individual trees within a species. It is important to note, however, that while highly significant differences were detected between species, there were also very significant differences amongst individual trees within a species. Without replicated sampling and an estimate of within-tree variation grossly misleading results could have been obtained, in which variation between individual trees of a species was mistaken for a specific difference. The analyses here presented emphasize the need in taxonomic work involving quantitative variables for experimental designs from which tree-to-tree variation can be adequately estimated. Indeed, in the early herbarium studies of the genus, much confusion arose from there being insufficient specimens for the botanist to be able to judge the extent of the morphological variation to be expected within a *Cupressus* species. It appears that a similar situation applies to their chemical makeup.

The mean values calculated for the four alkane component ratios are given in Table 2 together with a "least significant difference" at the 5 per cent probability level.

From the table, it is clear that *C. benthamii*, *C. lindleyi* and *C. lusitanica* differ only slightly and inconsistently in their leaf wax alkane components, and much less than they do from the

TABLE 2. MEAN RATIOS OF ALKANE COMPONENTS OF CYPRESS LEAF WAXES

Species	Ratio			
	C_{29}/C_{27}	C_{31}/C_{29}	C_{35}/C_{31}	$(C_{25}+C_{26})/C_{27}$
New World				
Cupressus arizonica	0 869	4·263	0·318	0·305
C. benthamii	1·417	2·613	5·000	1·389
C. lindleyi	1·551	3·084	4·055	0·985
C. macrocarpa	2·664	2·374	0·540	0·414
C. lusitanica	1·432	3·764	1·699	0·841
Old World				
C. funebris	3·743	0·317	5·225	1·380
C. torulosa	3·155	6·674	0·490	1·680
C. sempervirens	4·689	2·111	0·519	1·802
L.S.D. 5% probability	1·049	1·050	1·396	0·374

other species. This supports modern botanical opinion that they are only three forms of the one species—*C. lusitanica*. With regard to the remaining species, no one ratio identifies all successfully. Thus the C_{29}/C_{27} ratio fails to separate *C. arizonica* from the *C. lusitanica* group, or *C. funebris* from the other Old World species, but both can be immediately distinguished by reference to the C_{31}/C_{29} ratio. It was concluded that the minor alkane patterns can be used as a specific discriminant.

Discrimination of Old World and New World Groups

The alkane ratio $(C_{25}+C_{26})/C_{27}$ was selected as being the most promising for distinguishing Old and New World cypresses. In Old World species, the C_{25} and C_{26} alkanes are present in almost equal amounts and C_{27} is only slightly more abundant. The ratio $(C_{25}+C_{26})/C_{27}$ will therefore always be more than unity and will approach a value of 2·0 when the three alkanes are present in nearly equal quantities. The New World species, on the other hand, show much larger proportions of C_{27} alkane than they do C_{25}, and the amount of C_{26} is extremely small. In these species the ratio is less than unity (average 0·772; Table 2), whereas in Old World species it is 1·621. It is clear that this ratio separates the two groups, and *C. lusitanica* (ratio 0·841) belongs to the New World group. An analysis of variance of the data indicated that the two populations were distinct at the 99·9 per cent probability level. *C. lusitanica* data resembled the New World at the same probability level.

This result is in accordance with the majority botanical opinion, and with the findings of Erdtman[12] that the two groups of cypresses may be distinguished by the presence of manool and torulosic acid in the resins of Old World species, and their absence from New World species. L. J. Gough,[13] however, has unpublished data which indicate that there may be exceptions to Erdtman's distinction.

[12] H. ERDTMAN, in *Chemical Plant Taxonomy* (edited by T. SWAIN), pp. 96–123. Academic Press, London (1963).

[13] L. J. GOUGH (Borough Polytechnic, London), personal communication.

Intermediate Constitution of Leaf Wax Alkane Fractions of Hybrid Cypresses

Hybrid seedlings of *C. arizonica* ♀ × *C. lusitanica* ♂ and *C. lusitanica* ♀ × *C. macrocarpa* ♂ were obtained by controlled pollination. The hybrids were intermediate in growth and appearance between the parent species. Insufficient foliage could be taken from the small plants to sample individual trees, but three samples of the leaf wax of each hybrid were collected from the pooled leaves of five individuals. The chromatograms of the alkane components of the hybrid waxes are shown in Fig. 1, with similar chromatograms from the parent species. It is interesting to note that the characteristic features of the parent waxes appear in the hybrid waxes. A relatively large C_{35} peak, usual in *C. lusitanica*, appears in both hybrids. Similarly, relatively large C_{29} and C_{27} peaks, characteristic of *C. macrocarpa* and *C. arizonica* respectively, also appear on the chromatograms of their respective hybrids.

TABLE 3. COMPARISONS OF LEAF WAX ALKANES OF HYBRID AND PUTATIVE HYBRID CYPRESSES WITH WAX ALKANES OF THE PARENT SPECIES

Species	Mean values of alkane ratios			
	$\frac{C_{29}}{C_{27}}$	$\frac{C_{31}}{C_{29}}$	$\frac{C_{35}}{C_{31}}$	$\frac{C_{25}+C_{26}}{C_{27}}$
Cupressus arizonica	0·869	4·263	0·318	0·305
Hybrid ♀ *C. arizonica* × *C. lusitanica* ♂	2·544	2·458	0·723	0·342
Cupressus lusitanica	1·432	3·764	1·699	0·841
Hybrid ♀ *C. lusitanica* × *C. macrocarpa* ♂	3·395	2·875	0·928	0·594
Putative hybrids	1·863	2·395	1·143	0·540
Cupressus macrocarpa	2·664	2·374	0·540	0·414

(Hybrid values intermediate between parent species underlined).

The ratios of the alkane components were calculated for the hybrid waxes in the same way as for specific samples, but the mean values were not found to be intermediate in all cases. The discrepancy was thought to be due to the small number of samples available and to the small size of individual samples. This opinion was supported when the waxes of putative hybrid trees of *C. lusitanica* ♀ × *C. macrocarpa* ♂ were examined. Intermediate looking trees were selected in Kenya crops and confirmed as intermediate by biometric techniques similar to those used by Cousens[14] to identify hybrid oaks. Paired samples of leaf wax were obtained from each tree selected. Considerable variation was found between trees but the mean alkane ratios proved to be intermediate between the putative parent species. The alkane ratios found for hybrids and putative hybrids are compared with parent species means in Table 3.

Application of the Results

Having established that *Cupressus* species could be identified by their leaf wax alkane ratios, it remained to compare the Kenya strain of *C. lusitanica* with cypresses of certain identity. When this was done it was found that leaf wax of the Kenya strain did not differ

[14] J. F. COUSENS, *Watsonia* 5, 275 (1963).

significantly from either European or American strains of the species. The wax analyses showed no sign of hybridization with *C. macrocarpa* or other species, and provided valuable additional evidence for the conclusion reached from the morphological study by Dyson[6] that the Kenya preferred cypress has arisen by selection from *C. lusitanica* seed introductions, and is not the product of hybridization.

EXPERIMENTAL

Field Sampling

For specific samples, trees were selected in stands which had been raised from seed introduced to Kenya from the natural distribution area of the species concerned. In cases of doubt (*C. benthamii*), herbarium material was collected from the trees sampled and matched in the herbarium. Branches were collected from all parts of the crown of the tree and well mixed. Two foliage samples were drawn from each set of branches. The "A" sample was taken from the extreme tips of the current season's new growth on primary and second-order branches; the "B" sample was taken from young growth on third- and fourth-order branchlets. If any older material was collected in order to make up the required weight (about 50 g) it was invariably included in the "B" sample. The paired samples from each tree were stored green in polyethylene bags until required.

Chemical Examination

The procedures for isolating the alkane fraction of the leaf waxes and their analysis by gas–liquid chromatography have been fully described in the first paper of this series.[15]

Acknowledgement—We thank Dr. G. H. Freeman of the East African Agriculture and Forestry Research Organisation, Muguga, Kenya, for advice on sampling techniques and the statistical treatment of the analytical data.

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