Studies on Leaf Surface Lipid of Tobacco IV. Comparisons of Epicuticular Alkane Composition in *Nicotiana* Species

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Alkane composition was determined on the leaves of 52 *Nicotiana* species and 2 horticultural species. All *Nicotiana* species except *N. glauca* contained substantial amounts of branched (iso- and anteiso-) alkanes. *Nicotiana* species were divided roughly into six groups by their resemblance on alkane composition.

This classification system was correlated to the botanical classification.

The evolution of *Nicotiana* species was discussed on the basis of alkane composition.

The use of chemical constituents of plants as an aid to their classification is now a familiar concept. The use of epicuticular wax in plant taxonomy would seem to be advantageous in view of the almost universal occurrence of these coating and the fact that the wax is extracellular and almost certainly an end product insulated from the regular, essential metabolic functions of the plant. Above all, hydrocarbon mixture is very amenable to extract and rapid analysis by gas chromatography, and its very complexity serves as a positive advantage in providing a taxonomic "finger print." Attempting to test this approach, Eglinton and his colleagues examined the leaf waxes of closely related genera of the subfamily Sempervivoideae (Crassulaceae).^{1,2)} In another study, Borges del Castillo *et al.* examined representatives of the families Podocarpaceae, Araucaraceae and Cupressaceae.³⁾ These results showed that there are some correlations between carbon number distribution and botanical classification. Since the above mentioned pioneering studies, many studies on the epicuticular wax from different species have been conducted⁴⁾ and the data are still accumulating.^{5,6)}

On the other hand, additional investigations with a limited range of plants have shown that factors such as growing conditions and age of tissue can also influence the distribution of

wax components.^{7~9)} Although these results showed some difficulties for use of alkane composition in plant systematics, it is still an useful marker to distinguish plant species by the alkane composition, taking extent of the variation in consideration.

Nicotiana species have frequently been used for introduction of disease resistant character into commercial tobacco plants by breeding. Since epicuticular wax may serve to preserve the water balance and to prevent the mechanical damage or fungal and insect attack, it is of interest to study the components of epicuticular wax in *Nicotiana* species. This paper deals with the comparative studies on epicuticular alkane composition in *Nicotiana* species.

MATERIALS AND METHODS

Seeds of *Nicotiana* species were derived from self-pollinated flowers at the Iwata Tobacco Experiment Station. Plants were grown in a green house in 1978 and 1979 repeatedly. All leaves of 5 plants, from every stalk position appeared on the ground, were taken at 3 to 4 months after seeding, when the respective plants flowered. In the case of some species which were not flowered under the conventional green house condition, samples were taken at 4 months after seeding.

Leaf surface lipid was extracted by a short time dipping of the leaves into chloroform according to a previous report.¹⁰⁾ After concentration of the extract

in vacuo, alkane fraction was separated by a silica gel column according to a previous method.⁹⁾ Alkane components were analyzed by GC on a 3 m column of 1% OV 101. Each GC peak of typical samples was identified by GC-MS and co-chromatography with an authentic sample.

RESULTS

The present experiment used 52 out of 64 *Nicotiana* species and 2 horticultural species (*N. affinis* and *N. sandelae*). The plant names and relative composition of their alkane constituents are shown in Table I. Alkane components of the most species distributed into those which has carbon skelton number from 27 to 34.

The components are roughly divided into 2 groups, one is branched such as iso (2-methyl) and anteiso (3-methyl) series and the other is normal series. Since the 2 groups are biosynthesized from different precursors,¹¹⁾ respective analysis of the data for each group may be better way for species classification.

It has been known among various plants that *N. tabacum*, a commercial tobacco plant, is a species which contains branched alkane constituents in unusual high concentration.¹²⁾ Similar to *N. tabacum*, all *Nicotiana* species except for *N. glauca* had a substantial proportion of branched alkanes, and this may be a characteristic feature of *Nicotiana* species. In a previous paper,⁹⁾ we reported that composition of alkanes especially for the branched components in *N. tabacum* was versatile with leaf age and cultivation circumstances. Nevertheless, the alkane composition seems to be an useful marker for classification, taking the compositional fluctuation in consideration. To serve for analysis of the data, degree of annual difference of the alkane composition of typical species are parallely shown in Table I as 1978 and 1979 data.

As reported on many higher plants, normal series of alkane components in *Nicotiana* species also distributed mostly in those with odd number carbon skelton, especially in C_{29} , C_{31} and C_{33} . In respect to these 3 components, *Nicotiana* species may be divided into 4 groups,

where the first group consists of $n-C_{31}/n-C_{29} > 2$, $2 > n-C_{31}/n-C_{33} > 0.5$, and $n-C_{33}/n-C_{29} > 2$ (*n-A*), the second group $n-C_{31}/n-C_{29} > 2$, $n-C_{31}/n-C_{33} > 2$ and $2 > n-C_{33}/n-C_{29} > 0.5$ (*n-B*), the third group $2 > n-C_{31}/n-C_{29} > 0.5$, $n-C_{31}/n-C_{33} > 2$ and $n-C_{33}/n-C_{29} < 0.5$ (*n-C*), and the fourth group $n-C_{31}/n-C_{29} > 2$, $n-C_{31}/n-C_{33} < 0.5$ and $n-C_{33}/n-C_{29} > 2$ (*n-D*). The results are also shown in Table I.

All species except for *N. glauca* in Paniculatae section belong to *n-A*. *N. glauca* was a special species in 52 *Nicotiana* species so far examined in all, and contained about 90% of $n-C_{31}$ to total alkane content.

Species in Tomentosae section belong to either *n-A* or *n-B*. We classified *N. tabacum* cv. MC to be *n-B* in this experiment, but further experiments using 23 cultivars showed that these cultivars belong to either *n-A* or *n-B*.

All 3 species in Repandae section, *N. nudicaulis* in Nudicaulis section, *N. wigandoides* in Undulata section and *N. sylvestris* in Alatae section belong to *n-A*.

Although *N. rustica* in Rusticae section showed annual difference in alkane composition, we tentatively classified it to be *n-B* taking average number between 1978 and 1979 data. *N. trigonophylla* in Trigonophyllae section, *N. longiflora* and *N. plumbaginifolia* in Alatae section and *N. bigelovii* in Bigelovianae section belong to *n-B*.

In Sauveolentes species, 10 out of 17 species showed *n-A* and 5 species showed *n-B*. *N. simulans* may be *n-A*. *N. goodspeedii* showed a characteristic feature which does not contain $n-C_{33}$.

As above mentioned, 39 out of 52 species in *Nicotiana* showed either *n-A* or *n-B* character. Although *n-A* and *n-B* were classified in the present data analysis, it was occasionally hard to distinguish the group of some species which were on around the border of *n-A* and *n-B*. However, the above species could be clearly distinguished from the other two groups, *n-C* and *n-D*.

The third group, *n-C*, is consisted of 2 out of 3 species in Undulatae section and 2 out of 4 species in Acuminatae section. Another 2

species in Acuminatae section showed characteristic features for respective species, but we would like to join these 2 species into *n*-C because of their relatively high content in *n*-C₂₉ component. *N. clevelandii* in Bigelovinae section belong to this group.

The species which belong to *n*-D were only detected in Alatae section, where 6 including 2 horticultural species (*N. affinis* and *N. sandelae*) out of 9 Alatae species belong to *n*-D.

In regard to branched alkane composition, *Nicotiana* species may be divided into 4 groups, where the first group consists of $\text{iso-C}_{31}/\text{iso-C}_{29} > 2$ and $2 > \text{anteiso-C}_{32}/\text{anteiso-C}_{30} > 0.5$ (*b*-A), the second group $\text{iso-C}_{31}/\text{iso-C}_{29} > 2$ and $8 > \text{anteiso-C}_{32}/\text{anteiso-C}_{30} > 2$ (*b*-B), the third group $\text{anteiso-C}_{32}/\text{anteiso-C}_{30} < 0.5$ regardless with $\text{iso-C}_{31}/\text{iso-C}_{29}$ (*b*-C), and the fourth group $\text{iso-C}_{31}/\text{iso-C}_{29} > 8$ and $\text{anteiso-C}_{32}/\text{anteiso-C}_{30} > 8$ (*b*-D).

In the case where the species showed annual difference in the grouping, for example in *N. knightiana* or *N. rustica*, the average number of the two samples was taken for tentative decision to which group the species belong to.

Five species in Paniculatae section except for *N. glauca*, belong to *b*-A. *N. rustica*, *N. tabacum*, *N. trigonophylla*, 3 out of 9 species in Alatae section, 5 out of 17 species in Sauveolentes section also belong to *b*-A.

The second group, *b*-B, is consisted of all three species in Repandae section, *N. nudicaulis* in Nudicaulis section, and 9 out of 17 species in Sauveolentes section.

Contrary to the above two groups, the third group, *b*-C, is consisted of species in more restricted sections, that is, 2 out of 3 species in Undulatae section, all 2 species in Bigelovinae section and all 4 species in Acuminatae section. As an exception, *N. glutinosa* in Tomentosae section belong to this group.

As to the fourth group, *b*-D, 6 species in Alatae section, which classified as *n*-D, belong to this group. In addition to this, 3 species in Sauveolentes section also showed the similar character.

Although the above grouping is not rigid but still fluctual in some extent, the present results

may show that the classification of *Nicotiana* species by alkane composition has some correlation with the botanical classification.

DISCUSSION

In an authorized book "The Genus *Nicotiana*,"¹⁸⁾ Goodspeed proposed an evolution scheme of *Nicotiana* species (Table II). Consideration of the present data on the evolutionary aspect may be of interest, because each species seems to have its characteristic alkane composition or species in the same section have similar alkane composition.

N. rustica, which is being cultivated as a commercial tobacco in USSR, is known to be evolved after ancient hybridization of *N. paniculata* and *N. undulata*,¹³⁾ and showed an intermediate character in alkane composition between both progenitors. Previously, Dyson and Hervin¹⁶⁾ reported that hybrid plants of *Cypripedium* species produced an intermediate alkane constitution of their parent species. Suspecting from the results, alkane composition in *N. rustica* would be a reflection of its parent progenitors.

According to Goodspeed, 5 species in Tomentosae section (except for *N. kawakamii* which was a new species discovered in 1968) might be evolved from a pre-subgenic aggregate, pre-*Tabacum*. Although there are some species gap one another, these species showed generous similarity in alkane composition.

As described in Results, *N. tabacum* cultivars showed one of those characters which were *n*-A or *n*-B and *b*-A or *b*-B. Since these cultivars are the products after many times breedings not only in *N. tabacum* but also in some cases with the other *Nicotiana* species, we could not decide which cultivar is the typical *N. tabacum*. Unfortunately, it has not yet been detected wild *N. tabacum* in spite of enormous survey. Nevertheless, either of *N. tabacum* cultivar showed similarity in alkane composition with those in Tomentosae section. *N. tabacum* is known to be arisen after hybridization of *N. sylvestris* and *N. tomentosiformis*.^{14,15)} Although *N. sylvestris* is botani-

TABLE I. COMPOSITION OF EPICUTICULAR ALKANES IN NICOTIANA SPECIES

Section	Species	Sampling year	Alkane composition (% to total alkane content)												Type of composition				
			C ₂₇		C ₂₈		C ₂₉		C ₃₀		C ₃₁		C ₃₂				C ₃₃		C ₃₄
			i-a	n-	a-	n-	i-	n-	a-	n-	i-	n-	a-	n-	i-	n-	a-	n-	Normal alkane
Paniculatae	<i>N. glauca</i>	1979	—	—	—	—	—	—	—	—	1.2	88.2	0.6	1.3	—	8.2	—	—	? ^d
	<i>N. paniculata</i>	1979	—	—	—	0.4	1.9	8.3	5.1	3.2	12.9	27.6	9.1	3.8	6.2	21.0	0.6	—	n-A
	<i>N. knightiana</i>	1979	—	0.2	—	0.4	2.9	7.2	5.0	3.0	12.9	20.1	9.6	4.9	8.7	21.7	2.5	0.7	n-A
																	4.8	0.9	n-A
	<i>N. solanifolia</i>	1979	—	0.2	—	0.3	2.5	4.6	4.0	2.6	22.3	23.4	7.9	3.0	10.7	17.9	0.6	—	n-A
	<i>N. benavidesii</i>	1979	0.6	4.9	1.1	0.5	5.9	6.0	10.8	1.2	11.9	22.0	8.5	2.2	6.0	16.6	0.3	—	n-A
	<i>N. rainmondii</i>	1979	—	1.9	—	0.5	3.9	7.3	7.0	1.5	11.6	29.8	7.5	2.3	7.1	19.7	—	—	n-A
																			b-A
Rusticae	<i>N. rustica</i>	1979	—	0.6	—	0.6	5.3	10.8	3.4	2.8	14.4	36.4	2.5	2.5	3.9	16.9	—	—	n-B
		1978	—	1.9	—	1.0	8.3	10.4	7.1	4.4	13.8	19.5	6.1	5.5	7.4	14.9	—	—	n-B ^e
Tomentosae	<i>N. tomentosiformis</i>	1979	0.4	5.2	1.2	0.7	5.4	5.3	13.7	1.2	12.6	20.6	12.3	2.2	6.3	11.1	—	—	n-A
	<i>N. otophora</i>	1979	—	1.3	—	0.6	0.7	3.7	6.9	1.6	11.6	22.5	25.0	4.0	9.2	12.0	1.0	—	n-A
		1978	—	1.3	—	0.6	0.6	4.2	7.1	2.0	11.9	20.1	22.2	5.3	10.0	13.3	—	—	n-A
	<i>N. setchellii</i>	1979	—	2.9	—	0.8	4.3	10.7	10.2	2.4	12.3	32.4	7.2	2.5	3.7	10.2	—	—	n-B
		1978	—	2.8	—	—	4.2	11.3	9.7	2.6	12.2	33.5	6.7	2.5	3.5	10.1	—	—	n-B
	<i>N. glutinosa</i>	1979	—	1.9	0.3	0.8	8.7	8.4	16.4	2.1	15.5	24.3	5.7	2.4	4.3	8.1	—	—	n-B
		1978	—	1.7	—	—	8.7	8.6	16.8	2.4	15.5	26.6	4.8	2.2	3.6	7.5	—	—	n-B
	<i>N. kawakamii</i>	1979	0.5	9.1	0.8	1.6	8.0	9.4	10.5	2.4	14.7	23.7	5.6	2.3	3.2	5.8	—	—	n-B
<i>N. tomentosa</i>	1979	—	3.6	0.2	0.5	1.4	4.1	7.0	1.4	6.8	26.5	12.3	5.0	5.4	24.6	1.3	—	n-A	
Genuinae		1980	—	3.5	—	—	1.0	3.9	4.3	1.9	5.7	17.7	12.0	8.7	9.1	20.8	4.8	—	n-A
	<i>N. tabacum</i> (MC)	1979	0.2	6.8	0.1	1.6	0.1	7.4	11.0	2.3	11.1	25.3	15.7	3.2	3.6	9.5	—	—	n-B
Undulatae	<i>N. undulata</i>	1979	0.5	3.3	0.8	1.9	11.1	25.8	9.5	6.0	6.1	26.1	1.3	2.0	0.4	4.8	—	—	n-C
		1978	—	2.2	—	1.3	9.3	20.0	8.2	5.5	8.0	30.2	1.5	4.0	1.4	8.0	—	—	n-C
	<i>N. arentsii</i>	1979	—	1.3	—	2.0	9.9	22.7	9.5	6.3	11.7	24.6	3.1	2.2	1.4	5.4	—	—	n-C
		1978	—	—	—	1.5	5.3	20.3	7.7	6.2	13.0	29.2	3.8	3.9	1.9	6.5	—	—	n-C
	<i>N. wigandioides</i>	1979	—	0.6	—	0.6	7.3	8.0	6.1	2.5	6.0	32.7	9.5	3.7	3.5	23.2	—	—	n-A

TABLE 1. Continued

Section	Species	Sampling year	Alkane composition (% to total alkane content)														Type of composition																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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^a Following abbreviations were used; i⁻: iso (2-methyl), n⁻: normal and a⁻: anteiso- (3-methyl) homologues of alkane.

^b *N. affinis* is a horticultural species developed from *N. forgetiana*.

^c *N. sanderae* is a horticultural species produced by crossing of *N. alata* and *N. forgetiana*.

^d ? means the type is different from the category to any groups classified in this paper.

^e The type is decided from the average value from 1978 and 1979 data.

^f ( ) means the type is not the same but very similar with the type shown in the bracket.

TABLE II. THE EVOLUTIONARY SCHEME OF *Nicotiana* SPECIES PROPOSED BY GOODSPEED AND THE CLASSIFICATION BY ALKANE COMPOSITION

cally classified in Alatae section, it has also similar character in alkane composition with the species in Tomentosae section, but not with the majority of the Alatae species. Therefore it may be reasonable that *N. tabacum* has Tomentosae type alkane composition.

In Undulatae species, *N. undulata* and *N. arentsii* has similar alkane composition, while *N. wigandeoides* has quite different composition. Although these 3 species are botanically classified in the same section, *N. undulata* and *N. arentsii* were thought to be arisen after contact or pre-Rustica with pre-Petunioides aggregate, while *N. wigandeoides* was arisen after contact of pre-Tabacum and pre-Petunioides aggregate, and this may be the reason why *N. wigandeoides* has different alkane composition from the other two species. Goodspeed cited that *N. wigandeoides* is marked in similarities to Tomentosae section botanically, and the present result also showed that *N. wigandeoides* has similar alkane composition with Tomentosae species.

Species in Alatae section is thought to be products of pre-Petunioides aggregate. Above all, *N. sylvestris* ($n=12$) would be evolved the most anciently from 12 paired pre-Alatae race. What next occurred was the chromosome reduction, and *N. longiflora* and *N. plumbaginifolia* would be arisen ($n=10$). The final chromosome reduction in this section would be occurred producing another species in Alatae section ($n=9$). Effect of the chromosome reduction might reflect to the alkane composition, as the species with $n=9$ has quite different alkane composition from the other species.

Species in Sauveolentes section so far examined in all showed very low relative contents in iso- C_{29} . In this regard, these species are similar with Alatae species with $n=9$. Another chemotaxonomical similarity between the species in Sauveolentes and Alatae sections has been shown in the primary structure of Fraction 1 protein.¹⁵⁾ These results may imply that either Sauveolentes or Alatae species were

evolved from the same progenitor, pre-Petunioides, as Goodspeed proposed.

The present results showed close relation between alkane composition and evolutionary situation of *Nicotiana* species. However, we are still afraid of that the compatibility is fortuitous because alkane composition is very flexible with the environmental condition where plants grow. To confirm the hypothesis that alkane composition has correlation with evolution of species, more precise experiments under restricted condition must be necessary and our study is going to this direction.

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