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Specific mixtures of secretions from male scent organs of African milkweed butterflies (Danainae)

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SUMMARY

The abdominal androconial organs (hairpencils: male scent glands) of samples of ten African milkweed butterfly species (Lepidoptera: Danainae) belonging to *Danaus*, *Tirumala* and *Amauris*, including all nine species commonly encountered in Kenya, have been analysed by gas chromatography and mass spectrometry. A total of 214 compounds have been identified, belonging to 14 chemical classes: hydrocarbons, alcohols, aldehydes, ketones, esters, lactones, carboxylic acids, oxidized carboxylic acids, aromatics, derivatives of pyrrolizidine alkaloids (PAs), monoterpenes, sesquiterpenes, other terpenoids and tetrahydrofurans. Various compounds only rarely or never found in insects before, including some previously unknown in nature, are present in the hairpencils.

Excluding the numerous tetrahydrofurans, which were not investigated systematically, the number of compounds ranges from 12–59 per species. All ten species have distinct mixtures of volatiles, including, in all cases, species-specific compounds (autapomorphies). In addition, the co-occurrence of compounds between species (synapomorphies) exhibits a strongly hierarchical chemo-taxonomic pattern which has been demonstrated to be largely consistent with a previous cladistic analysis based on adult morphology. The potential significance of these findings in relation to chemical communication and speciation in these mimetic butterflies is discussed.

1. INTRODUCTION

The milkweed butterflies (Lepidoptera: Danainae) are one of the best known of all groups of tropical Lepidoptera, being remarkable for their involvement in mimicry, chemical defence, chemical communication and migration (Ackery & Vane-Wright 1984; Boppré 1984). The Afrotropical Region has a total of 22 species of these butterflies, of which 15 occur on the African mainland. In Kenya, from where most of the samples reported on here were obtained, a maximum of 11 species occur, representing the genera *Danaus*, *Tirumala* and *Amauris*.

These danaine butterflies are faced with a communication problem: Müllerian and Batesian mimicry affects all of the species in question. In Kenya, the most notable examples include complexes relating to the patterns of *Danaus chrysippus* (at least 6 spp.), *Amauris niavius* (at least 10 spp.) and *Amauris echeria* (more than 10 spp.). Some polymorphic species, such as *Hypolimnas anthedon* (Nymphalinae) and *Papilio dardanus* (Papilionidae), mimic two or three of these danaine models; in other cases, species appear to have patterns intermediate between models (e.g., *Graphium leonidas*, Papilionidae), and there are even day-flying moths involved (e.g. *Nyctemera* spp., Arctiinae) (see, for example, Eltringham 1910; Owen 1974; Pinhey 1977; plate 2 in Vane-Wright & Boppré 1993). The potential confusion for intraspecific recognition and communication among these visually orientating insects is presumed to be overcome by elaborate chemical communication systems (see § 4*d*).

The chemistry of pheromones of male danaine butterflies has been studied quite extensively over the last two decades (for review and references see Ackery & Vane-Wright (1984)). Interest has largely been focused on the dihydropyrrolizine components of the odour bouquets because their biosynthesis depends on precursors which the adult males have to gather from plants (cf. § 4*d*). However, although very important in mate choice, dihydropyrrolizines only quantitatively are the major components of danaine pheromone bouquets since these compounds show little or no differentiation among sympatric danaine species and, although great variation in the amount of dihydropyrrolizines typically occurs, there is little evidence to suggest that the different PA derivatives provide species-specific signals.

It has been known for a long time that danaine hairpencils secrete odours which, to the human nose, are strong but pleasant and appear to be species-specific (Latter 1935; Seibt *et al.* 1972; D. Schneider, personal communication). For *Amauris ochlea*, Petty *et al.* (1977) elucidated the structure of some of the hairpencils volatiles, and for *A. niavius* Meinwald *et al.* (1974) reported the presence of 35 components, 33 of

which, however, were not identified. The carboxylic acids from the hairpencils of several *Amauris* and the volatile compounds from *A. echeria* have recently been described by Schulz *et al.* (1988*a*, *b*). For some Australasian species, single volatiles have been characterized (see § 4).

Here, we report on detailed chemical analyses of extracts of the abdominal androconial organs ('hairpencils'; for chemistry see Schulz (1987), for morphology see Boppré & Vane-Wright (1989)) of ten species of Danainae belonging to the three genera found in Africa and, for the first time, an attempt is made to examine the chemical basis of sexual communication in a community of aposematic butterflies. The numerous hairpencil volatiles identified (see table 3 for systematic listing, table 6 for grouping) are related to morphological, ethological and ecological data and serve to discuss evolutionary (including cladogenetic) aspects.

2. MATERIAL AND METHODS

Our studies involve *Amauris* (*Amaura*) *albimaculata* Butler, *A.* (*Amaura*) *damocles* (Fabricius), *A.* (*Amaura*) *echeria* (Stoll), *A.* (*Amaura*) *hecate* (Butler), *A.* (*Amaura*) *ochlea* (Boisduval), *A.* (*Amauris*) *niavius* L. (subspecies *niavius* and *dominicanus* Trimen), *A.* (*Amauris*) *tartarea* Mabille, *Danaus* (*Anosia*) *chrysippus* (L.), *Tirumala* (*Elsa*) *formosa* (Godman), and *T.* (*Tirumala*) *petiverana* (Doubleday). Adult males of these species (except *A. damocles*) were collected in the field in Kenya (East Africa), mostly near Kwale (Coast Province) and Kakamega (Western Province); samples of *A. damocles*, which does not occur in East Africa, as well as additional samples of *hecate*, *niavius*, *tartarea*, *chrysippus* and *petiverana*, were obtained from Porto Novo (Republic de Bénin) and in the vicinity of Kpalime (Republic de Togo), West Africa (cf. table 2).

The hairpencils were protruded by manual pressure, removed with forceps, touched on absorbent paper to remove excess haemolymph and kept in groups of 3–30 in vials containing pentane (Merck, Uvasol). In Germany, samples were stored at −70°C. For analysis each sample was ground up using a glass rod, and the homogenate filtered through a cotton plug. The extracts were used for micro-reactions or concentrated to an appropriate volume for use in gas chromatographic (GC) and mass spectrometric (MS) analyses. Hairpencil components were identified by comparing their mass-spectra and gas-chromatographic retention times with those of reference samples. In addition, chemical transformations of crude extracts were performed to facilitate identification of unknown compounds. For a detailed analysis of 2,5-dialkyl-tetrahydrofurans (THFs), a hairpencil extract of *niavius* was separated by chromatography over a small silica gel column (5 mm × 50 mm, Merck, mesh 230–400) using hexane as eluent; fractions containing THFs were combined prior to GC analysis.

(*a*) *Analytical procedures*

Chemical analyses were carried out with a Carlo-Erba Fractovap 2101 gas chromatograph with flame ionization detector and split or on-column injection, using concentrated crude or chemically modified extracts (i.e. equivalents to the androconial secretion of 0.1–2.0 males). Separations were performed using fused silica and glass capillary columns coated with one of the following phases: SE-30, SE-54, CP-Sil-8-CB, FS-FFAP-CB, WG-11Q. Helium served as the carrier gas.

Low and high resolution mass spectra (70 eV) were obtained with a VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph, and with a Varian MAT 311 A instrument coupled to a Carlo-Erba Fractovap 2101 gas chromatograph.

Numerous components of hairpencil extracts of *ochlea* were assessed quantitatively by comparing peak areas in gas chromatograms against an internal standard (hexadecane; no response factors determined).

(*b*) *Chemical transformations*

To obtain saturated compounds, parts of an extract were hydrogenated using palladium on charcoal (Francke *et al.* 1989*a*). Carboxylic acids, esters, ketones and aldehydes were sometimes transformed into respective alcohols with lithium aluminium hydride (Francke *et al.* 1989*a*). To improve elution properties of fatty acids, methylations were performed with freshly prepared diazomethane (Schulz *et al.* 1988*a*). In mono-unsaturated compounds, double-bond positions were determined by treating extracts with dimethyldisulfide (DMDS) (Buser *et al.* 1983; Francis & Veland 1982), while in polyunsaturated acids this was achieved by reacting crude extracts with oxalylchloride followed by treatment with 3-pyridinemethanol (Harvey 1984). To reveal presence of polyalcohols, extracts were treated with 50 µl N-methyl-N-(trimethylsilyl)-trifluoracetamide for 15 min at room temperature, followed by evaporation of the solvent and excess reagent.

(*c*) *Syntheses of reference compounds*

Various carboxylic acids, (*E*,*Z*)-2,6-nonadienal and (*Z*)-6-nonen-4-olide were synthesized as described in Schulz *et al.* (1988*a*). Esters of aliphatic acids were synthesized by standard procedures. (*E*,*E*)-2,6-dimethyl-8-hydroxy-2,6-octadienal was prepared by selenium dioxide oxidation of geranyl acetate, followed by pyridinium dichromate oxidation and hydrolysis. Dihydroactinidiolide was prepared according to the procedure of Nickson (1986). The dihydroedulans as well as the corresponding epoxide, 9,10-epoxy-1,3,7,7-tetramethyl-2-oxabicyclo[4.4.0]decane, were prepared as described in Francke *et al.* (1989*a*). Hexahydrofarnesylacetone was obtained by hydrogenation of farnesylacetone. Different 2,5-dialkyltetrahydrofurans were prepared by two subsequent alkylations of furan (Brandsma & Verkruijsse 1986), followed by hydrogenation over palladium/charcoal. Hydroxydanaidal was prepared by hydrolysis of heliotrine or a mixture of axillarine-axillaridine, followed

Table 1. *Sample sizes and provenance of African danaine hairpencils analysed*

	number of hairpencils	number of extracts analysed	years of collection	origin
D. chrysippus	45	2	'86	Kenya: Coast
	15	2	'89–'90	Bénin, Togo
T. petiverana	19	3	'84–'85	Kenya: Coast, Kakamega
	15	1	'90	Bénin
T. formosa	35	6	'85–'86	Kenya: Kakamega
A. tartarea	17	6	'84–'86	Kenya: Kakamega
	5	2	'89–'90	Bénin, Togo
A. niavius	258	12	'84–'86	Kenya: Coast, Kakamega
	16	2	'89–'90	Togo
A. echeria	547	14	'84–'86	Kenya: Kakamega
A. hecate	6	4	'84–'86	Kenya: Kakamega
	7	1	'90	Togo
A. albimaculata	59	6	'84–'86	Kenya: Kakamega
A. damocles	51	6	'90–'91	Bénin, Togo
A. ochlea	245	11	'85–'87	Kenya: Coast

by oxidation using manganese dioxide (Bell & Meinwald 1986).

3. RESULTS

(*a*) *Compounds analysed in detail*

Eighty-one samples with androconia of 1370 specimens were analysed (table 1). The general pattern (cf. table 2) involves a more or less large number of individual compounds (12–59) in any given species. Table 3 lists all 152 compounds identified, with the exception of THFs but including 16 as yet unidentified components, sorted and labelled according to established or assumed biosynthetic relationships, i.e. they have been classified into acetogenins, mevalogenins, alkaloids and aromatic compounds. Acetogenins were further differentiated into hydrocarbons, alcohols, aldehydes, ketones, esters, lactones, carboxylic acids and oxidized carboxylic acids; mevalogenins were classed as monoterpenes, sesquiterpenes and other terpenoids. Figure 1*a–j* shows representative gas chromatograms of extracts from hairpencils of all ten species, while figure 2 demonstrates the diversity of molecular structures encountered.

All acetogenic substances exhibit a straight carbon chain and occur as saturated or unsaturated compounds. In addition, some saturated methyl-branched alkanes are found, as well as oxidized fatty acids and acyclic ketones. The aromatic compounds involve alcohols, aldehydes, ketones, carboxylic acids, esters and ethers. Alkaloids occur as dihydropyrrolizine derivatives. Terpenes appear as mono- and dioxygenated monoterpenes, sesquiterpenes, and – probably originating from carotenoids (Francke *et al.* 1989*a*) – C_9-, C_{12}-, C_{13}- or C_{18}-compounds.

The information compiled in table 3 reflects exclusively those compounds found in repeated analyses based on several samples, from at least two separate years and/or two locations. Thus, we are confident that the data are reliable in the sense that any contaminants have been excluded. The absolute as well as the relative amounts of compounds may vary within a species, most notably affecting dihydropyrrolizines. The latter exhibit great quantitative variation according to individual uptake of precursors; for example, *hecate* hairpencils collected in 1984 showed no danaidone (J1), whereas samples taken in the following years had significant amounts. This effect is caused by different access during the year to plants containing precursors. Ninety per cent of the compounds identified were reproducibly present in samples taken at different times. This consistency can be explained by the discreteness of the hairpencils, which makes them comparatively easy to prepare cleanly. Strikingly, few differences occur between samples from east and west Africa: in *chrysippus* from east Africa tiny amounts of danaidal (J2) have been found repeatedly; two samples of this species from west Africa lacked danaidal entirely.

Table 4 shows the absolute amounts of the major compounds for *ochlea*. Because of the large differences in quantity of individual compounds encountered among species, table 4 indicates only presence or absence, but figure 1*a–j* provides some indication of differences in peak area and thus relative quantities. Despite the fact that many compounds appeared to occur in only minute amounts, in almost all cases these substances were reliably and repeatably detectable.

(*b*) *Tetrahydrofurans*

In addition to the more or less volatile compounds mentioned above, a number of compounds eluting after squalene also occur on the hairpencils. In *niavius*, for example, 62 2,5-dialkyltetrahydrofurans (THFs) were identified (cf. figure 3, table 5). The chain lengths vary from 27–35 carbon atoms. Both *cis*- and *trans*-THFs occur, very often in complex mixtures.

Table 2. *Number of compounds found in each species of African danaine investigated (table 1), and the proportions of different chemical classes (table 3) represented by those components*

(*t*, total number of compounds per species, and in set of all ten species combined; *u*, number of compounds unique to each species, and only represented once in all ten species considered; *u/t*, percentage of total for species unique to that species; *u/a*, number of unique compounds per species as a percentage of total set of all 168 compounds; A–N, number of compounds per class (table 3: A = hydrocarbons, etc.) as a percentage of total number of compounds per species (bold numbers: highest proportions other than carboxylic acids (G) or unidentified compounds (N)). Tetrahydrofurans (table 5) were not investigated systematically, and are therefore not included here.)

	t	*u*	*u/t*	*u/a*	A	B	C	D	E	F	G	H	I	J	K	L	M	N
D. chrysippus	19	10	53	6.0	**16**	0	0	0	0	0	26	0	5	11	**26**	0	0	6
T. petiverana	12	4	33	2.4	0	0	0	0	0	**25**	50	0	8	8	0	0	0	8
T. formosa	12	1	8	0.6	**25**	8	0	0	**17**	0	50	0	0	0	0	0	0	0
A. tartarea	32	2	6	1.2	**47**	0	0	0	0	0	47	0	**6**	0	0	0	0	0
A. niavius	48	19	40	11.3	**52**	0	0	2	2	0	19	0	**6**	4	4	2	2	8
A. echeria	52	21	40	12.5	**13**	0	0	0	6	0	31	**15**	10	4	6	4	4	4
A. hecate	43	18	42	10.7	5	0	0	**19**	0	2	58	**9**	0	2	0	0	2	2
A. albimaculata	40	3	8	1.8	**5**	0	**5**	**5**	0	2	65	2	2	3	0	0	2	8
A. damocles	59	15	25	8.9	3	2	5	3	0	5	51	**10**	5	3	0	0	**7**	5
A. ochlea	45	3	7	1.8	**7**	0	2	4	0	**7**	62	2	4	2	0	4	0	4
	168	96	26	5.7	17	1	1	3	2	4	46	4	5	4	4	1	2	6
	totals										means							

Although THFs can be readily recognized by their mass spectra, exhibiting prominent ions formed by α-cleavage next to the tetrahydrofuran ring and subsequent loss of water, THFs were not analysed in all species because of their low volatility and late elution in gas chromatography.

(*c*) *Species-specificity of hairpencil bouquets*

As can be seen from tables 2 and 3, all species investigated have distinctive hairpencil bouquets, composed of 12–59 different substances from the total of 168 compounds listed in table 3. If THFs are included, the number of compounds per bouquet may be as many as 110, as exemplified by *niavius* (cf. table 5). All species have unique compounds (i.e. compounds not found in the other species), ranging from just one (out of 12) in *formosa*, up to 21 (out of 52) in *echeria*. Expressed as a proportion of the total number of compounds per species, the uniques make up 6–53% of the bouquet components; as a proportion of all 168 compounds, they make up 0.6–12.5% (table 2).

If the bouquet components are considered proportionally with respect to the chemical classes listed in table 3, clear species-specific profiles are apparent. Disregarding the dominant carboxylic acids (G; but see also § 4) and the unidentified substances (N), all species (except *niavius* and *tartarea* with respect to each other) are separable by combinations of most strongly represented chemical classes (table 2):

lactones	*Tirumala petiverana*
hydrocarbons + esters	*Tirumala formosa*
hydrocarbons + monoterpenes	*Danaus chrysippus*
hydrocarbons + aromatics	*Amauris tartarea*
hydrocarbons + aromatics	*Amauris niavius*
hydrocarbons + oxidized carboxylic acids	*Amauris echeria*
hydrocarbons + aldehydes + ketones	*Amauris albimaculata*
hydrocarbons + lactones	*Amauris ochlea*
oxidized carboxylic acids + ketones	*Amauris hecate*
oxidized carboxylic acids + terpenoids	*Amauris damocles*

With respect to the overall composition of the specific bouquets, the carboxylic acids are found in all samples and make up, on average, 46% of the bouquet components (table 2). *Amauris albimaculata* (65%) and *ochlea* (62%) are relatively the most rich in carboxylic acids, while *niavius* (19%) is the poorest. The only other class richly represented overall is the hydrocarbons (average: 17%), but in this case the specific variation is greater, rising to more than 50% of the bouquet composition in *niavius*, but lacking altogether in *petiverana*. The least well represented classes are the alcohols, aldehydes and sesquiterpenes (1% average composition each), but these are all included in the list of most narrowly distributed chemical classes (table 2): alcohols (2 out of 10 species), aldehydes (3/10), esters (3/10), monoterpenes (3/10) and sesquiterpenes (3/10). Representation of chemical classes per species ranges from as few as three in *tartarea* (hydrocarbons, carboxylic acids and aromatics only; no unknowns) to as many as ten in *damocles* (tables 2 and 3). Species and higher-taxon specificities are highlighted in detail below, where the patterns of occurrence are discussed with respect to systematic and functional relationships.

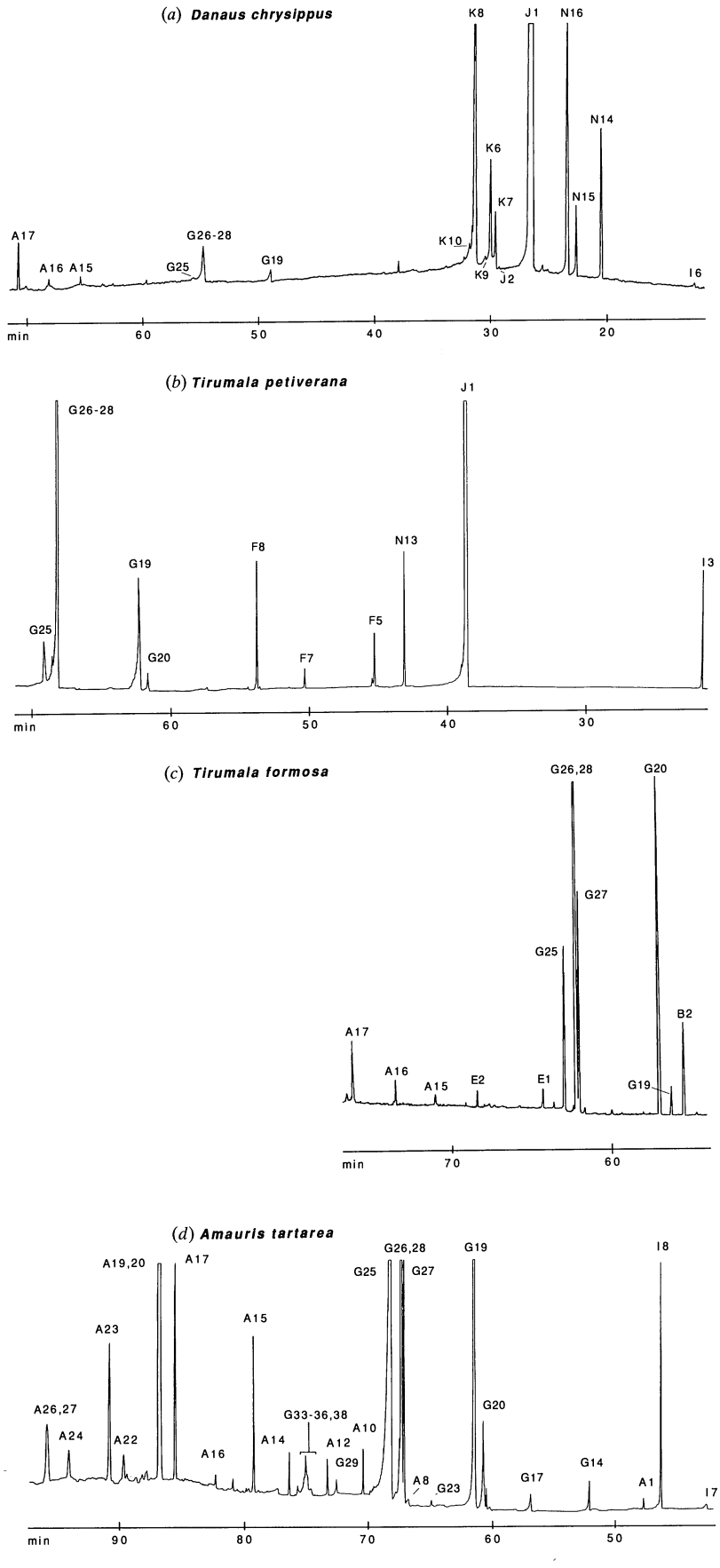

Figure 1*a–d*. Legend on p. 167.

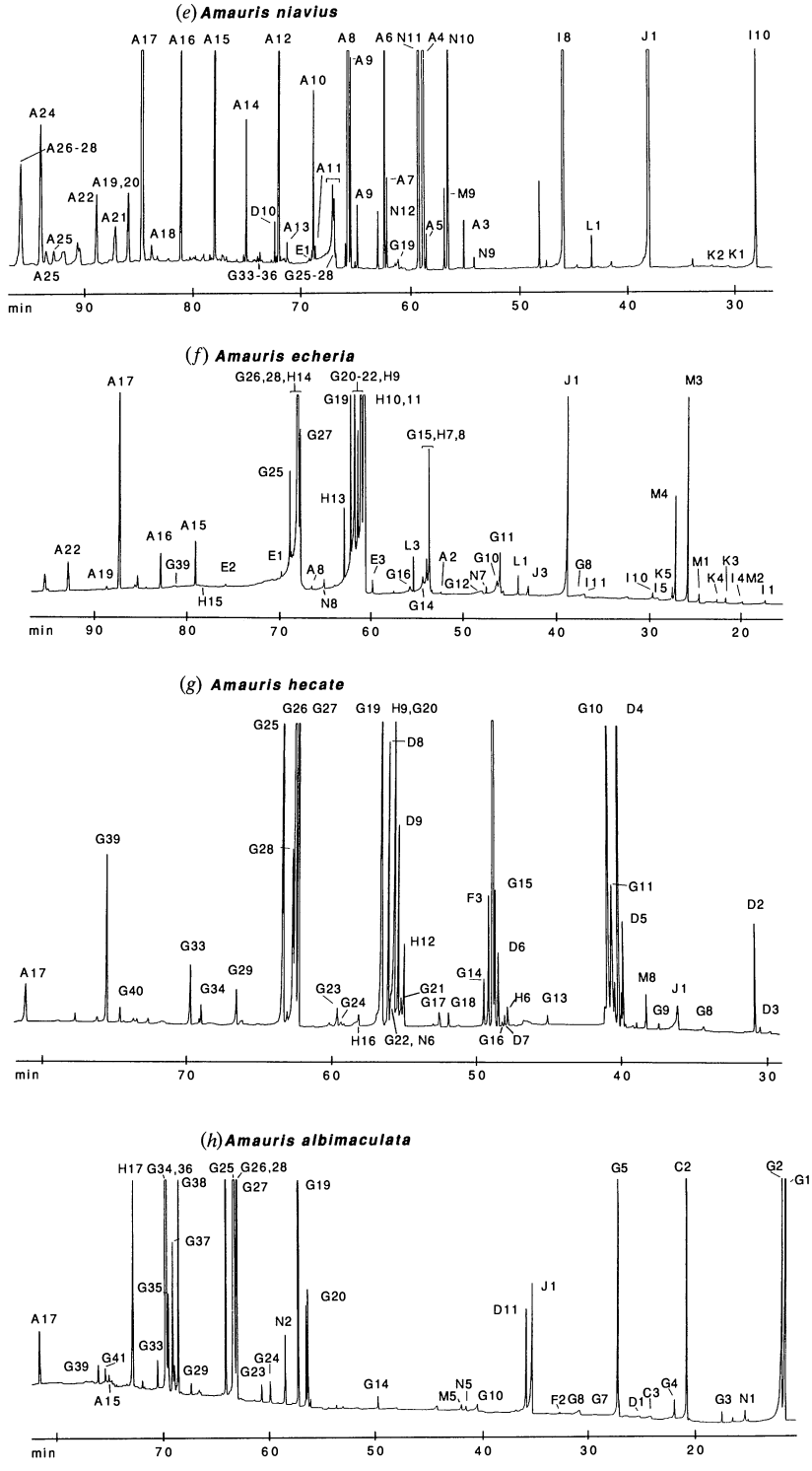

Figure 1*e–h*. Legend on facing page.

4. DISCUSSION

Some of the species reported on here have been investigated before for hairpencil compounds. Petty *et al.* (1977) found C2, D11, G2, I10, I11, J1 and octanal in *ochlea*. Except for octanal, we obtained the same results, but in addition identified some 40 more components. Meinwald *et al.* (1974) found danaidone (J1) in *ochlea*, *albimaculata*, *echeria*, *niavius*, *petiverana*, and *chrysippus* but not in *tartarea* or *formosa*. In *tartarea*, Meinwald *et al.* (1974) identified 3,4-dimethoxyacetophenone (I8) only; this compound had been found by these authors also in *niavius*, together with at least 33 other unknown compounds. The present work confirms these results, including the restriction of I8 to *tartarea* and *niavius* (table 3; figure 1*a–j*). (*E*)-2,6-Dimethyl-6-octen-1,8-diol (K8) has been found in *D. chrysippus* (Meinwald *et al.* 1971), together with the

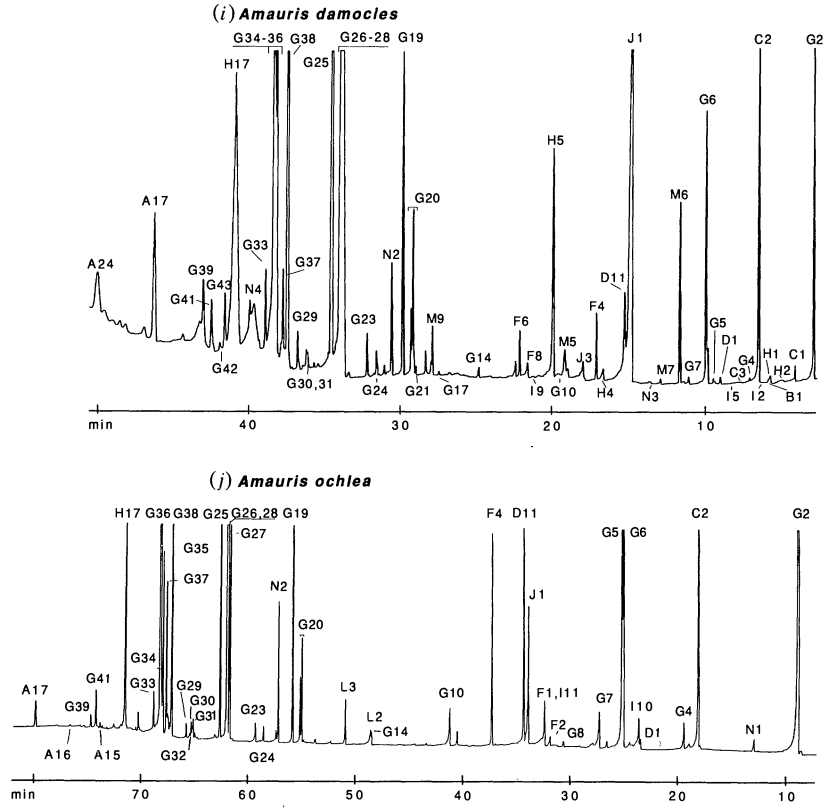

Figure 1. Representative gas chromatograms of hairpencil extracts of the 9 species of Danainae found in Kenya, plus *A. damocles* from Bénin. (*a*) *Danaus chrysippus*; (*b*) *Tirumala petiverana*; (*c*) *T. formosa*; (*d*) *Amauris tartarea*; (*e*) *A. niavius*; (*f*) *A. echeria*; (*g*) *A. hecate*; (*h*) *A. albimaculata*; (*i*) *A. damocles*; (*j*) *A. ochlea*. GC conditions: (*a*) 30 m SE-54-CB, 60–300°C at 3°C min^{-1}; (*b*) 50 m SE-54, 6 min at 60°C, then to 280°C at 3°C min^{-1}; (*c*) 50 m SE-54, 60–280°C at 3°C min^{-1}; (*d,e*) 60 m DB-5, 6 min at 60°C, then to 290°C at 3°C min^{-1}; (*f–h, j*) 60 m DB-5, 6 min at 60°C, then to 290°C at 3°C min^{-1} (methylated extract); (*i*) 25 m CP-Sil-8-CB, 5 min at 60°C, then to 280°C at 5°C min^{-1}.

analog bishomoterpene (*E,E*)-3,7-dimethyl-2,6-decadien-1,10-diol, which was absent in the insects investigated by us.

(*a*) *Distribution of compounds identified in other insects*

The small dihydropyrrolizine alkaloids (J1–J3) have so far not been identified from natural sources other than lepidopteran androconia (species belonging to other danaine genera, Ithomiinae, Arctiidae and Ctneuchidae; for references see, for example, Ackery & Vane-Wright (1984); Boppré (1990); Schulz (1987) and unpublished results; F. Schmidt, unpublished results).

The hydrocarbons identified are commonly found in insect cuticle (Lockey 1988; Blomquist *et al.* 1987). Nevertheless, some of the compounds identified by us have been reported to act as pheromones: A20 is a weak aphrodisiac in the sulphur butterfly *Colias eurytheme* (Grula *et al.* 1980). A26 and A27 are reported to act synergistically with the female copulation-release pheromone, callosobrucic acid, in the Azuki bean weevil *Callosobruchus chinensis* (Tanaka *et al.* 1981). The hydrocarbons A12, A15, A20, A26 and A27 are constituents of fly pheromones (Fletcher & Bellas 1988).

The occurrence of fatty acids (G) and their oxidized analogs (H6–H17) has been discussed by Schulz *et al.* (1988*a*). 4-Hydroxybutanoic acid (H1), the diacids butanedioic (H2), octanedioic (H3), and nonanedioic acid (H5) as well as the respective aldehyde 9-oxononanoic acid (H4), have not been reported from any exocrine gland in insects before. Nevertheless, H4 and H5 as well as nonanoic acid (G5) and the respective aldehyde (C2) are typical autoxidation products of unsaturated fatty acids. However, only minor amounts may be formed by this process, and their presence in some species in quite substantial amounts suggests specific biosynthesis.

Octanol (B1) has been found in *Duforea* bees (Tengö *et al.* 1985) and several other Hymenoptera (see Wheeler & Duffield 1988). Hexadecanol (B2) has been reported from androconia of the lycaenid butterfly *Lycaeides argyrognomon* (Lundgren & Bergström 1975). B2, the corresponding acetate E1 and eicosyl acetate (E2) are saturated analogs of well-known female sex pheromone components (Arn *et al.* 1992).

Nonanal (C2) has also been reported from *Lycaeides argyrognomon* (Lundgren & Bergström 1975), and is a female attractant in the androconia of the waxmoth, *Galleria mellonella* (Leyrer & Monroe 1973). The lilac aldehyde (*E,Z*)-2,6-nonadienal (C3) and the respective acid (G7) have not been identified in any other insect, while the related lactone (F2) is present in androconia of two species of *Aphomia* waxmoths (Kuwahara 1980; Kunesch *et al.* 1987).

Saturated methylketones are commonly found in a

Table 3. *Compounds identified from hairpencil extracts of African milkweed butterflies*

(co#, code for chemical class and compound; ch#, numbers for chemical characters as used in cladistic analysis (cf. Vane-Wright *et al.* 1992, table 3); Ao, *Amauris ochlea*; Ad, *Amauris damocles* (included in addition to the Kenyan spp. to document cladistic analysis in Vane-Wright *et al.* (1992), and for comparison with *A. ochlea*; see discussion); Aa, *Amauris albimaculata*; Ah, *Amauris hecate*; Ae, *Amauris echeria*; An, *Amauris niavius*; At, *Amauris tartarea*; Tf, *Tirumala formosa*; Tp, *Tirumala petiverana*; Dc, *Danaus chrysippus*. AG, acetogenins; AC, aromatic compounds; AL, alkaloids; MG, mevalogenins.)

co#		name of compound	ch#	Ao	Ad	Aa	Ah	Ae	An	At	Tf	Tp	Dc
hydrocarbons (AG)													
A	1	hexadecene								*			
A	2	heptadecane						*					
A	3	octadecane							*				
A	4	nonadecane							*				
A	5	nonadecene							*				
A	6	eicosane							*				
A	7	eicosene							*				
A	8	heneicosane	1					*	*	*			
A	9	heneicosene							*[a]				
A	10	docosane	2						*	*			
A	11	docosene							*				
A	12	tricosane	3						*	*			
A	13	tricosene							*				
A	14	tetracosane	4						*	*			
A	15	pentacosane	5	*		*		*	*	*	*		*
A	16	hexacosane	6	*				*	*	*	*		*
A	17	heptacosane	7	*	*	*	*	*	*	*	*		*
A	18	heptacosene							*				
A	19	11-methylheptacosane	8					*	*	*			
A	20	13-methylheptacosane	9						*	*			
A	21	11,15-dimethylheptacosane							*				
A	22	octacosane	10					*	*	*			
A	23	13-methyloctacosane								*			
A	24	nonacosane	11		*		*		*	*			
A	25	nonacosene							*[a]				
A	26	11-methylnonacosane	12						*	*			
A	27	13-methylnonacosane	13						*	*			
A	28	15-methylnonacosane							*				
alcohols (AG)													
B	1	octanol			*						*		
B	2	hexadecanol											
aldehydes (AG)													
C	1	octanal			*								
C	2	nonanal	14	*	*	*							
C	3	(*E*,*Z*)-2,6-nonadienal	15		*	*							
ketones (AG)													
D	1	decan-2-one	16	*	*	*							
D	2	undecan-2-one					*						
D	3	10-undecen-2-one					*						
D	4	tridecan-2-one					*						
D	5	12-tridecen-2-one					*						
D	6	pentadecan-2-one					*						
D	7	14-pentadecen-2-one					*						
D	8	heptadecan-2-one					*						
D	9	16-heptadecen-2-one					*						
D	10	heneicosan-2-one							*				
D	11	*cis*-jasmone	17	*	*	*							
esters (AG)													
E	1	octadecyl acetate	18					*	*		*		
E	2	eicosyl acetate	19					*			*		
E	3	methyl (*E*)-7-oxo-11-tetradecenoate						*					
lactones (AG)													
F	1	2-nonen-4-olide		*									
F	2	(*Z*)-2,6-nonadien-4-olide	20	*		*							

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Table 3. *Continued*

co#		name of compound	ch#	Ao	Ad	Aa	Ah	Ae	An	At	Tf	Tp	Dc
F	3	5-dodecanolide					*						
F	4	11-dodecanolide	**21**	*	*								
F	5	5-dodecen-11-olide										*	
F	6	13-tetradecanolide			*								
F	7	tetradecenolide										*	
F	8	5-tetradecen-13-olide[b]	**22**		*							*	
carboxylic acids (AG)													
G	1	hexanoic acid				*							
G	2	(*Z*)-3-hexenoic acid	**23**	*	*	*							
G	3	heptanoic acid				*							
G	4	octanoic acid	**24**	*	*	*							
G	5	nonanoic acid	**25**	*	*	*							
G	6	(*Z*)-6-nonenoic acid	**26**	*	*								
G	7	(*E*,*Z*)-2,6-nonadienoic acid	**27**	*	*	*							
G	8	decanoic acid	**28**	*		*	*	*					
G	9	undecanoic acid					*						
G	10	dodecanoic acid	**29**	*	*	*	*	*					
G	11	dodecenoic acid	**30**				*	*[c]					
G	12	dodecadienoic acid						*					
G	13	tridecanoic acid					*						
G	14	tetradecanoic acid	**31**	*	*	*	*	*		*			
G	15	tetradecenoic acid	**32**				*	*[c]					
G	16	tetradecadienoic acid	**33**				*	*					
G	17	pentadecanoic acid	**34**		*		*			*			
G	18	pentadecadienoic acid					*						
G	19	hexadecanoic acid	**35**	*	*	*	*	*	*	*	*	*	*
G	20	hexadecenoic acid	**36**[d]	*[c]	*	*[a]	*	*[c]		*	*	*	
G	21	hexadecadienoic acid	**38**		*		*[a]	*[a]					
G	22	hexadecatrienoic acid	**39**				*	*					
G	23	heptadecanoic acid	**40**	*	*	*	*			*			
G	24	heptadecenoic acid	**41**	*	*	*	*						
G	25	octadecanoic acid	**42**	*	*	*	*	*	*	*	*	*	*
G	26	octadecenoic acid	**43**	*[c]	*	*	*	*[c]	*	*	*	*	*
G	27	octadecadienoic acid	**44**	*	*	*	*	*[a]	*	*	*	*	*
G	28	octadecatrienoic acid	**45**	*	*	*	*	*	*	*	*	*	*
G	29	nonadecanoic acid	**46**	*	*	*	*			*			
G	30	nonadecenoic acid	**47**	*	*								
G	31	nonadecadienoic acid	**48**	*	*								
G	32	nonadecatrienoic acid		*									
G	33	eicosanoic acid	**49**	*	*	*	*		*	*			
G	34	eicosenoic acid	**50**	*	*	*	*		*	*			
G	35	eicosadienoic acid	**51**	*	*	*			*	*			
G	36	11,14,17-eicosatrienoic acid	**52**	*	*[c]	*			*[c]	*[c]			
G	37	8,11,14,17-eicosatetraenoic acid	**53**	*	*[c]	*							
G	38	5,8,11,14,17-eicosapentaenoic acid	**54**	*	*[c]	*				*[c]			
G	39	docosanoic acid	**55**	*	*	*	*	*					
G	40	docosenoic acid					*						
G	41	13,16,19-docosatrienoic acid	**56**	*	*[c]	*							
G	42	docosatetraenoic acid			*								
G	43	docosapentaenoic acid			*								
oxidized carboxylic acids (AG)													
H	1	4-hydroxybutyric acid			*								
H	2	butanedioic acid			*								
H	3	octanedioic acid			*								
H	4	9-oxononanoic acid			*								
H	5	nonanedioic acid			*								
H	6	5-oxododecanoic acid					*						
H	7	7-oxododecanoic acid						*					
H	8	7-oxo-11-dodecenoic acid						*					
H	9	7-oxotetradecanoic acid	**57**				*	*					
H	10	(*E*)-7-oxo-11-tetradecenoic acid						*					
H	11	(*Z*)-7-oxo-11-tetradecenoic acid						*					
H	12	7-oxo-13-tetradecenoic acid					*						
H	13	(*E*)-7-oxo-11,13-tetradecadienoic acid						*					

(*continued overleaf*)

Table 3. *Continued*

co#		name of compound	ch#	Ao	Ad	Aa	Ah	Ae	An	At	Tf	Tp	Dc
H	14	9-oxohexadecanoic acid						*					
H	15	11-oxooctadecanoic acid						*					
H	16	7-hydroxytetradecanoic acid					*						
H	17	9-hydroxyoctadecanoic acid	**58**	*	*	*							
aromatic compounds (AC)													
I	1	benzaldehyde	**59**					*					
I	2	benzoic acid	**59**		*				*				
I	3	2-phenylethanol	**60**									*	
I	4	phenylacetaldehyde	**60**					*					
I	5	2-phenylacetic acid	**60**		*	*		*					
I	6	acetophenone											*
I	7	acetovanillone	**61**							*			
I	8	3,4-dimethoxyacetophenone	**61**						*	*			
I	9	3,4-dimethoxybenzoic acid	**61**		*								
I	10	methyl salicylate	**62**	*				*	*				
I	11	eugenol	**63**	*				*					
PA-derivatives (AL)													
J	1	danaidone		*	*	*	*	*	*			*	*
J	2	danaidal											*
J	3	hydroxydanaidal			*			*					
monoterpenes (MG)													
K	1	neral							*				
K	2	geranial							*				
K	3	*cis*-5-(1-hydroxy-1-methylethyl)-2-methyl-2-vinyltetrahydrofuran						*					
K	4	*trans*-5-(1-hydroxy-1-methylethyl)-2-methyl-2-vinyltetrahydrofuran						*					
K	5	3-hydroxy-2,2,6-trimethyl-6-vinyl-tetrahydropyran						*					
K	6	(*E*)-2,6-dimethyl-5-octen-1,8-diol[2]											*
K	7	(*Z*)-2,6-dimethyl-5-octen-1,8-diol[2]											*
K	8	(*E*)-2,6-dimethyl-6-octen-1,8-diol											*
K	9	(*Z*)-2,6-dimethyl-6-octen-1,8-diol											*
K	10	(*E*,*E*)-2,6-dimethyl-8-hydroxy-2,6-octadienal											*
sesquiterpenes (MG)													
L	1	(*E*,*E*)-α-farnesene	**64**					*	*				
L	2	(*E*,*E*)-α-farnesol	**64**	*									
L	3	methyl (*E*,*E*)-farnesenoate	**64**	*				*					
other terpenoids (MG)													
M	1	isophorone						*					
M	2	β-phorone						*					
M	3	4-oxoisophorone						*					
M	4	2,2,6-trimethylcyclohexan-1,4-dione						*					
M	5	dihydroactinidiolide	**66**		*	*							
M	6	dihydroedulan I			*								
M	7	dihydroedulan II			*								
M	8	(1*S**,3*R**,6*S**,9*S**,10*R**)-9,10-epoxy-1,3,7,7-tetramethyl-2-oxabicyclo-[4.4.0]decane					*						
M	9	hexahydrofarnesylacetone	**65**		*				*				
unknown compounds[f]													
N	1	B81, M110	**67**	*		*							
N	2	M266	**68**	*	*	*							
N	3	B144, M172			*								
N	4	B201			*								
N	5	B91, M172				*							
N	6	B68					*						
N	7	B57						*					
N	8	BM220						*					
N	9	B82							*				

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Table 3. *Continued*

co#		name of compound	ch#	Ao	Ad	Aa	Ah	Ae	An	At	Tf	Tp	Dc
N	10	B82							*				
N	11	B82							*				
N	12	B82							*				
N	13	B55, M198										*	
N	14	B43, M155											*
N	15	B95, M168											*
N	16	B43, M157											*

[a] Mixture of two or more double bond regioisomers.
[b] Tentatively identified by MS only.
[c] Position of double bonds of monunsaturated fatty acids were investigated in *A. ochlea* and *A. echeria* only: while *A. ochlea* contains mostly one regioisomer, in *A. echeria* a complex mixture of isomers is found (cf. Schulz *et al.* 1988*a*).
[d] Character 37 (9-hexadecenoic acid) in Vane-Wright *et al.* (1992, table 3) is here included within hexadecenoic acid (character 36).
[e] Position of double bonds undetermined.
[f] Base peak (B) and presumed molecular peak (M) in mass spectra of unknown compounds.

variety of Hymenoptera (Blum 1981). In contrast, the ω-unsaturated methylketones D3, D5, D7, and D9 are only rarely encountered in insects: 10-undecen-2-one (D3) has been identified from *Duforea* bees (Tengö *et al.* 1985), 12-tridecen-2-one (D5) from termites (Prestwich & Collins 1982), and 14-pentadecen-2-one (D7) from the ant *Myrmecia nigriceps* (Jackson *et al.* 1990). The corresponding 16- heptadecen-2-one (D7) has not been reported from nature before. The cyclic ketone *cis*-jasmone (D11) is not known from any other lepidopteran androconia, but the closely related esters methyl jasmonate and methyl epijasmonate are courtship pheromones of male *Grapholita molesta* moths (Baker *et al.* 1981; Nishida *et al.* 1982).

The six-membered-ring-lactone 5-dodecanolide (F3) is known from some Hymenoptera (Jackson *et al.* 1990; W. Francke personal communication). 11-Dodecanolide (F4), exhibiting a twelve membered ring, is known from another danaine, *Euploea sylvester* (Schulz *et al.* 1988*b*). The other macrolides (F4–F8) have not been reported before from Lepidoptera, but were identified in *Cryptolestes* beetles (Wong *et al.* 1983; Millar *et al.* 1985*a*, *b*).

Several male moths use the aromatic compounds I1–I4 as courtship pheromones (Bestmann *et al.* 1977), but the compounds I5–I11 have not been reported from androconia of other species.

The widespread terpenes geranial (K1) and neral (K2) have been identified from scent scales of male *Pieris* butterflies and in various Hymenoptera (cf. Wheeler & Duffield 1988; Francke 1991). The cyclic linalooloxides (K3–K5) have not been reported from any other insect before. The terpene diols K8 and K9 are typical components of the hairpencil secretions of *Danaus* (Meinwald *et al.* 1971; Edgar 1982; Schulz 1987; Francke *et al.* 1989*a*). The regioisomers K6 and K7 have not previously been found in nature; the related alcohol K10, not found in insects until now, may be a biogenetic precursor.

(*E*,*E*)-α-Farnesene (L2) is widespread in insects, and has been identified as the main compound from the androconia of the swift moth *Hepialus humuli* (Schulz *et al.* 1990). (*E*,*E*)-α-Farnesol (L2) has been

Table 4. *Results of quantitative determination of a selection of compounds found in hairpencil extracts of Amauris ochlea (μg per male)*

A15	pentacosane	0.06
A16	hexacosane	trace
A17	heptacosane	1.50
C2	nonanal	2.06
D1	decan-2-one	0.01
D11	*cis*-jasmone + J1 danaidone	1.12
F1	2-nonen-4-olide + I11 eugenol	0.06
F2	(*Z*)-2,6-nonadien-4-olide	0.04
F4	11-dodecanolide	0.12
G2	(*Z*)-3-hexenoic acid	23.04
G4	octanoic acid	0.02
G5	nonanoic acid	2.76
G6	(*Z*)-6-nonenoic acid	
G7	(*E*,*Z*)-2,6-nonadienoic acid	0.08
G8	decanoic acid	trace
G10	dodecanoic acid	0.06
G14	tetradecanoic acid	0.02
G19	hexadecanoic acid	0.32
G20	hexadecenoic acid	0.38
G23	heptadecanoic acid	0.10
G24	heptadecenoic acid	0.08
G25	octadecanoic acid	2.98
G26	octadecenoic acid	74.66
G27	octadecadienoic acid	
G28	octadecatrienoic acid	
G29	nonadecanoic acid	0.44
G30	nonadecenoic acid	
G31	nonadecadienoic acid	
G32	nonadecatrienoic acid	
G33	eicosanoic acid	58.26
G34	eicosenoic acid	
G35	eicosadienoic acid	
G36	11,14,17-eicosatrienoic acid	
G37	8,11,14,17-eicosatetraenoic acid	
G38	5,8,11,14,17-eicosapentaenoic acid	
G39	docosanoic acid	0.10
G41	13,16,19-docosatrienoic acid	1.14
J17	9-hydroxy octadecanoic acid	0.96
I10	methyl salicylate	0.02
L2	(*E*,*E*)-α-farnesol	0.08
L3	methyl (*E*,*E*)-farnesenoate	0.10
N1	B81, M110	0.04
N2	M266	0.16

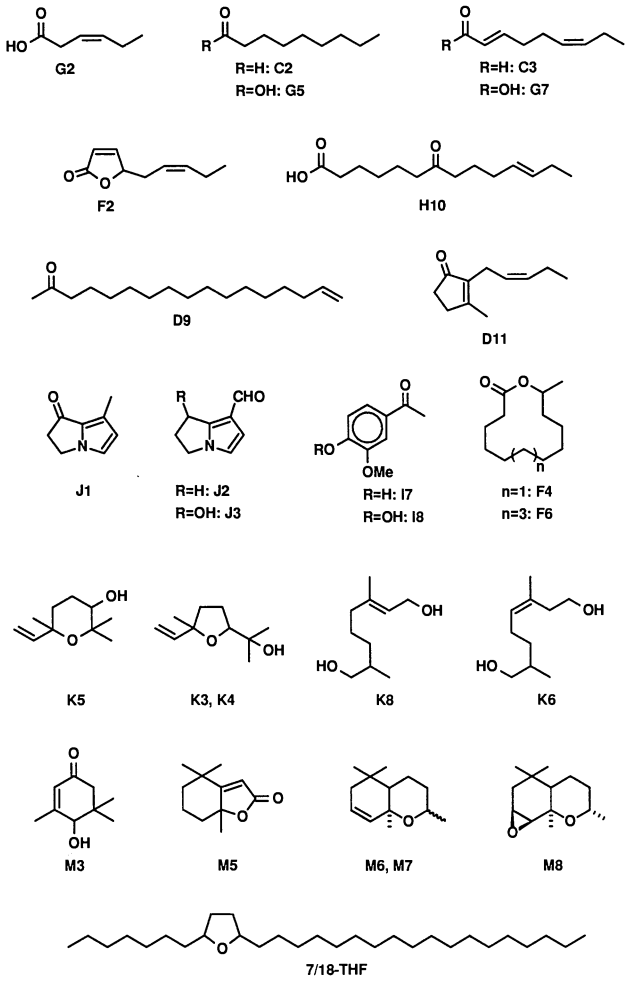

Figure 2. Molecular structures of a selection of compounds encountered in extracts of hairpencils of danaine butterflies to demonstrate their diversity. Labelling according to co# in table 3.

reported from many bees and ants (Francke 1991). The corresponding methyl ester L3 is related to juvenile hormones (Slama *et al.* 1974, pp. 137–193), but has not been identified from insects before.

Epoxytetrahydroedulan (M8) has been found in *hecate* in traces only, and is the main component of the volatile secretion of some *Euploea* species, together with the dihydroedulans M6 and M7 which are possibly the biogenetic precursors of M8 (Francke *et al.* 1989*b*).

The phorone M2 has been found in male bark beetles (*Ips typographus*; Birgersson *et al.* 1984) and occurs together with its isomer M1 in the defensive secretion of a grasshopper (Eisner *et al.* 1971); their oxidized analogs M3 and M4 are known from the danaine *Euploea sylvester* (Schulz *et al.* 1988*b*). The corresponding alcohol of hexahydrofarnesylacetone (M9) has been identified from androconia of the pyralid moth *Eldana saccharina* (Burger *et al.* 1985). Dihydroactinidiolide (M5) is a component of the queen recognition pheromone of the fire ant *Solenopsis invicta* (Rocca *et al.* 1983).

The THFs were also identified in many other species of butterflies investigated by us, including *Catopsilia* (Pieridae), and a variety of nymphalids: *Argynnis*, *Heliconius* (Heliconiinae), *Euploea* (Danainae), and several Ithomiinae (S. Schulz, unpublished results), independent of androconial organs. They occur in wing extracts of both sexes, and may therefore be common cuticle constituents. Takabayashi & Takahashi (1986*a*, *b*) also found them in surface extracts of larvae of *Pseudaletia separata* (Noctuidae), where they act as kairomones for a parasitic wasp.

(*b*) *Biological significance of compounds identified*

The secretions of lepidopteran androconia are known to be made of a great range of compounds belonging to many different classes such as acetogenins, mevalogenins, aromatic compounds, and alkaloids (cf. Schulz 1987). All these classes are represented in the hairpencils of the danaines investigated, which show the largest number of compounds (up to 110) identified from androconia so far.

Like hydrocarbons and many longer chain carboxylic acids, the THFs may represent another class of compounds typical for insect cuticle, at least in the Lepidoptera, because we identified them in many other lepidopteran species (above). Nevertheless, in principle it seems possible that all compounds identified could be pheromones. Many compounds identified have low volatility, but this should not be a limitation because all species analysed produce pheromone-transfer-particles which are stuck onto the female antennae during courtship (see § *d*), so that compounds with low volatility could be perceived.

On the other hand, it is unlikely that all compounds are signal transmitters. Many other functions are possible, such as solvents required to make effective pheromone formulations, glue (as proposed for a terpenoid related to K6–K9 found in *Danaus gilippus*: Pliske & Eisner 1969; Schneider & Seibt 1969), or as protection agents against microorganisms. For example, L3 proved to have microbicidal activity (S. Schulz, unpublished results).

Danaidone (J1) has been shown to be a courtship pheromone in *D. gilippus* (Pliske & Eisner 1969), while the other alkaloids (J2 and J3) have been shown to be pheromones of some arctiid moths (cf. Boppré 1990; § 4*d* below). While the dihydropyrrolizine alkaloids are typical for the danaines as a whole, the many other components may confer species specificity. If these compounds are produced by the butterflies themselves, they require substantial biosynthetic effort, as for example D1–D11, F1–F8, G2, G6, G7, G34–G38, G41–G43, H6–H17, I7–I9, K6–K10, M5–M8. It seems unlikely that all of these components are present just as byproducts of general metabolic pathways in the butterfly hairpencils, having no special function. If some of these compounds, which are similar in some respect to flower scents, are taken up from plants, as proposed for M6 and M7 (Francke *et al.* 1989*a*), it also seems unlikely that they are stored specifically in the hairpencils without use. In addition, the complex distribution of different types of hairs in the hairpencils (Boppré & Vane-Wright 1989) may suggest a far more complex use of hairpencils and odour bouquets than so far known (cf. Boppré 1990).

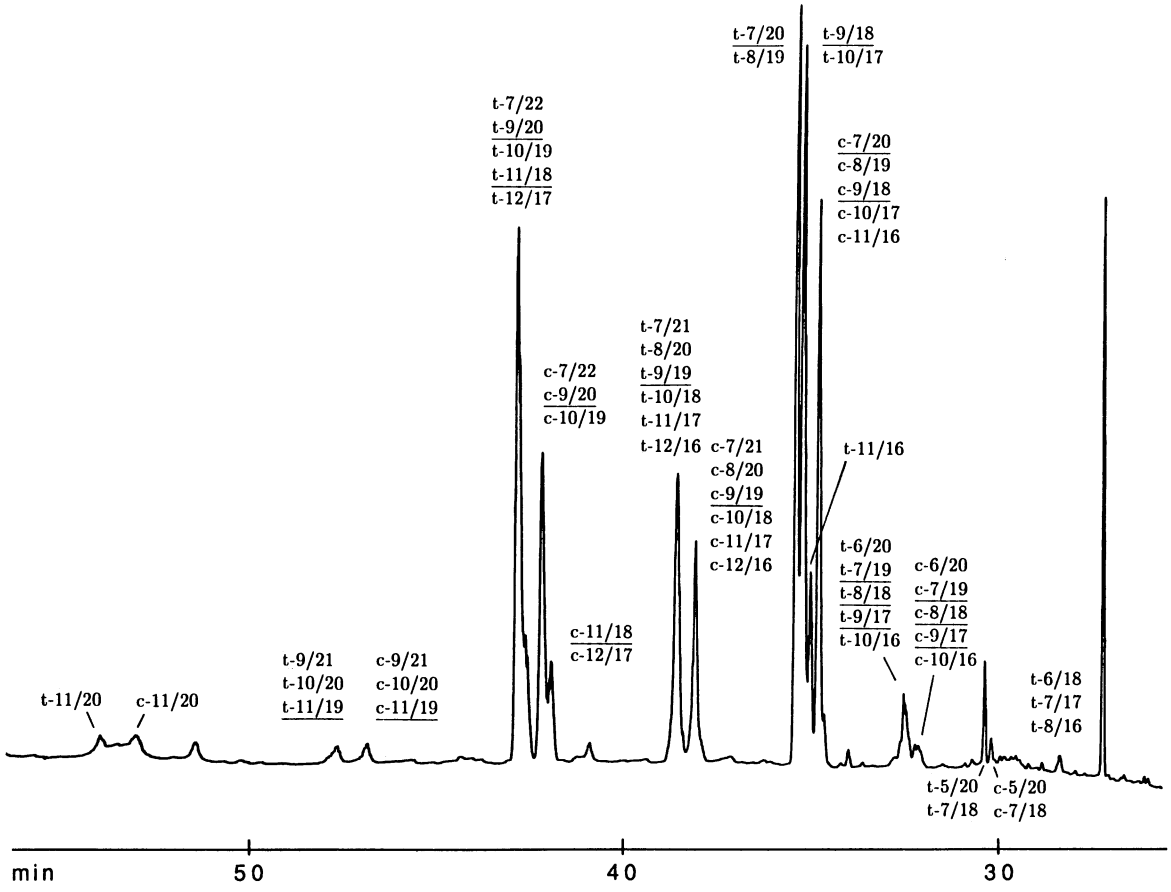

Figure 3. Gas chromatogram of 2,5-dialkyltetrahydrofuran fractions of hairpencil extracts of *Amauris niavius*. GC condition: 25 m CP-Sil-8-CB, 200–300°C at 5°C min^{-1}.

(*c*) *Species-specificity and systematic relationships*

(i) *Chemical autapomorphies and species-specificity*

Of the nine Kenyan danaines investigated, all have at least one relatively abundant hairpencil compound not found in the others (figure 1*a–j*; cf. table 3):

Danaus chrysippus	K6–K8, N14–16
Tirumala petiverana	F5, I3, N13
Tirumala formosa	B2
Amauris tartarea	A23
Amauris niavius	A4, A6, N10, N11
Amauris echeria	H10, H11, H13, M3, M4
Amauris hecate	D2, D4, D5, D8, D9, F3, H12
Amauris albimaculata	G1
Amauris ochlea	F4, G6

In the case of *tartarea*, two hydrocarbons (A19, A20: 11- and 13-methylheptacosane) which are present in large amounts are otherwise only found as traces in two other African danaine species, and it consistently lacks PA-derivatives in all samples analysed (a peculiarity also found in *formosa* in this study, but not in analyses of *formosa* carried out by W. Schäfer (unpublished results)). The two compounds noted for *ochlea*, F4 and G6, recur in the closely related west African species *damocles*, but they are not found in any of the Kenyan species investigated.

Thus, without exception, there is at least one major chemical substance in the hairpencil bouquet of each Kenyan species not found, or only found in small quantities, in the others. Such constituents could act as species-specific signals or markers sufficient to distinguish each danaine species within Kenya. However, cladistic analyses of the chemical data suggest other possibilities.

(ii) *Chemical synapomorphies and group-membership*

If at each speciation event, old signal compounds were abandoned in favour of new or unique substances, cladistic analysis of individual scent-gland chemicals would reveal only a 'bush' or totally unresolved tree, with autapomorphies for each of the species, but no indication of relationships between species (unless chemical transformation series were postulated). Such a pattern of replacement might be inferred from the work of Grula & Taylor (1979) on the inheritance of male pheromones in two semi-

Table 5. *2,5-Dialkyltetrahydrofurans identified in hairpencil extracts of* Amauris niavius

(Numbers indicate the side chain length of the THFs, e.g. 7/22 means 2-docosyl-5-heptyltetrahydrofuran. Major compounds are underlined. Each compound occurs as a mixture of *cis*- and *trans*-isomers.)

5/18	6/18	7/17	8/16	5/20	7/18	6/20	7/19	8/18
9/17	10/16	7/20	8/19	9/18	10/17	11/16	7/21	8/20
9/19	10/18	11/17	12/16	7/22	9/20	10/19	11/18	12/17
9/21	10/20	11/19	11/20					

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species of *Colias* (Pieridae) (see also Sappington & Taylor 1990*a–c*).

As we have just discussed, there is evidence that species-specific chemicals do occur in the majority of the danaine species studied here. However, if instead of abandoning old signal substances during speciation, novel substances were simply added to the old, we would expect to find not only complex 'bouquets' of chemicals within each species, but we would also be able to recover the cladistic relationships, of groups within groups, from an analysis of synapomorphies (unique co-occurrences: homologies) among the scent-gland chemicals alone. Such an additive model should be distinguishable from a subtractive model, in which an originally complex signal is successively simplified during speciation, or one in which signal specificity is achieved through alterations to the relative amounts of substances. In the case of an additive model, cladistic analysis would accurately reconstruct the phylogenetic relationships of all species involved, and reveal a pattern of larger and larger numbers of compounds at successive nodes. With a subtractive model, if loss apomorphies (reversals) are permitted in the analysis, the phylogenetic relationships will again be fully recovered, but with a pattern of decrease in number of compounds at successive nodes. If, on the other hand, functional specificity is achieved through variations in the relative amounts of substances, cladistic analysis of the chemical data coded as presence–absence will fail to recover any pattern of phylogenetic relationship, producing an unresolved bush, as when each species is marked only by unique substances (Vane-Wright & Boppré 1993).

In the present case, the data for individual chemical components from the ten African species investigated have been analysed cladistically by Vane-Wright *et al.* (1992). The 68 chemical characters (table 3: ch# 1–68) analysed alone produced a strongly hierarchical pattern of relationships close to, but not identical with expectations based on cladistic analysis of 32 morphological characters. An analysis of the chemical and morphological data combined produced a resolution of one of the morphological trees, and Vane-Wright *et al.* (1992) argued that this solution (figure 4) should be taken as the standard for evaluation of the chemical data alone. In the next section the outstanding features of the chemical data set are diagnosed against this tree, one node at a time. In a further analysis, we aggregated the majority of the identified individual chemical compounds into 27 biosynthetically related chemical groups (table 6), and these have also been diagnosed against the standard tree (this procedure is justified because a cladistic analysis of the 27 chemical groups taken as characters plus the 32 morphological characters used by Vane-Wright *et al.* (1992) produces an identical standard topology).

(iii) *Diagnosis of major features of the chemical hierarchy*

No single chemical compound forms a convincing synapomorphy for all Kenyan Danainae (node 1, figure 4). Only PA-derivatives are generally characteristic, but these are variable and proved to be entirely lacking in *formosa* and *tartarea*. Of these two species, *formosa* can almost certainly produce danaidone (see § 4*c*(i) above), but *tartarea* could be a genuine exception. Group 3 compounds (table 6: alkanes longer than docosane) occur in all ten species investigate except *petiverana*, while group 14 carboxylic acids (table 6: fatty acids between eicosanoic and tridecanoic acids) are found in all of them (tables 3 and 6).

There is also no convincing chemical synapomorphy for the three species of Danaina (node 2: *Danaus chrysippus*, *Tirumala petiverana* and *T. formosa*) nor, within this grouping, the two *Tirumala* species (node 3). *D. chrysippus* does have unique components, however, including three unknown substances (N12–N14), acetophenone (I6), danaidal (J2) and some dioxygenated monoterpenes (K6–K9). The same or related compounds have been found in other *Danaus* species (S. Schulz, unpublished results), but none of these butterflies occurs on the African mainland. Of the two *Tirumala* species, *petiverana* does have three unusual macrolides (F5, F7, F8, of which the last recurs in *damocles*), but only in very small amounts, together with unknown N13 which seems to be a macrolide too. In *T. formosa* hairpencils only low amounts of volatiles are present compared to other species; nevertheless, hexadecanol (B2) is a main component which, although it occurs quite widely in nature, is not found in any other of the African danaines, and could have a specific signal function for this butterfly.

The genus *Amauris* (node 4) also lacks an individual chemical synapomorphy, but can be grouped by the presence of fatty acids longer than nonadecanoic acid (group 15). However, some of the individual compounds among these interesting unsaturated C_{20}- acids (prostaglandin precursors: see Schulz *et al.* 1988a) almost have the status of synapomorphies. For example, all but *echeria* among the seven *Amauris* species investigated have the saturated analog eicosanoic acid. This compound has yet to be found in any other danaine species (S. Schulz, unpublished results). *A. echeria*, together with several other *Amauris*, produces docosanoic acid (one C_2 unit longer than eicosanoic acid). Given the overall picture of saturated fatty acid occurrence in *Amauris* (table 3), it is conceivable that *echeria* does produce eicosanoic acid, but only in amounts below the detection limit of our analyses. In addition, it is possible that the straight-chain hydrocarbons of groups 2 and 4 (table 6) were originally characteristic of *Amauris*, but have been lost subsequently in the common ancestor of *hecate* + *albimaculata* + *damocles* + *ochlea* (node 7).

Subgenus *Amauris* (node 5) is supported by a high incidence of hydrocarbons (25 in *niavius*, 15 in *tartarea*), compared with no more than seven in other African species investigated, including a group of alkenes shorter than tricosane (group 1). Although all of these compounds are generally widespread in insect cuticle, six are restricted in the data set to the two species of subgenus *Amauris*, and two of these compounds (A26, A27: 11- and 13-methylnonacosane) have not been recorded from other Danainae (S. Schulz, unpublished results). In addition, the aromatic I8 uniquely occurs as a major component in

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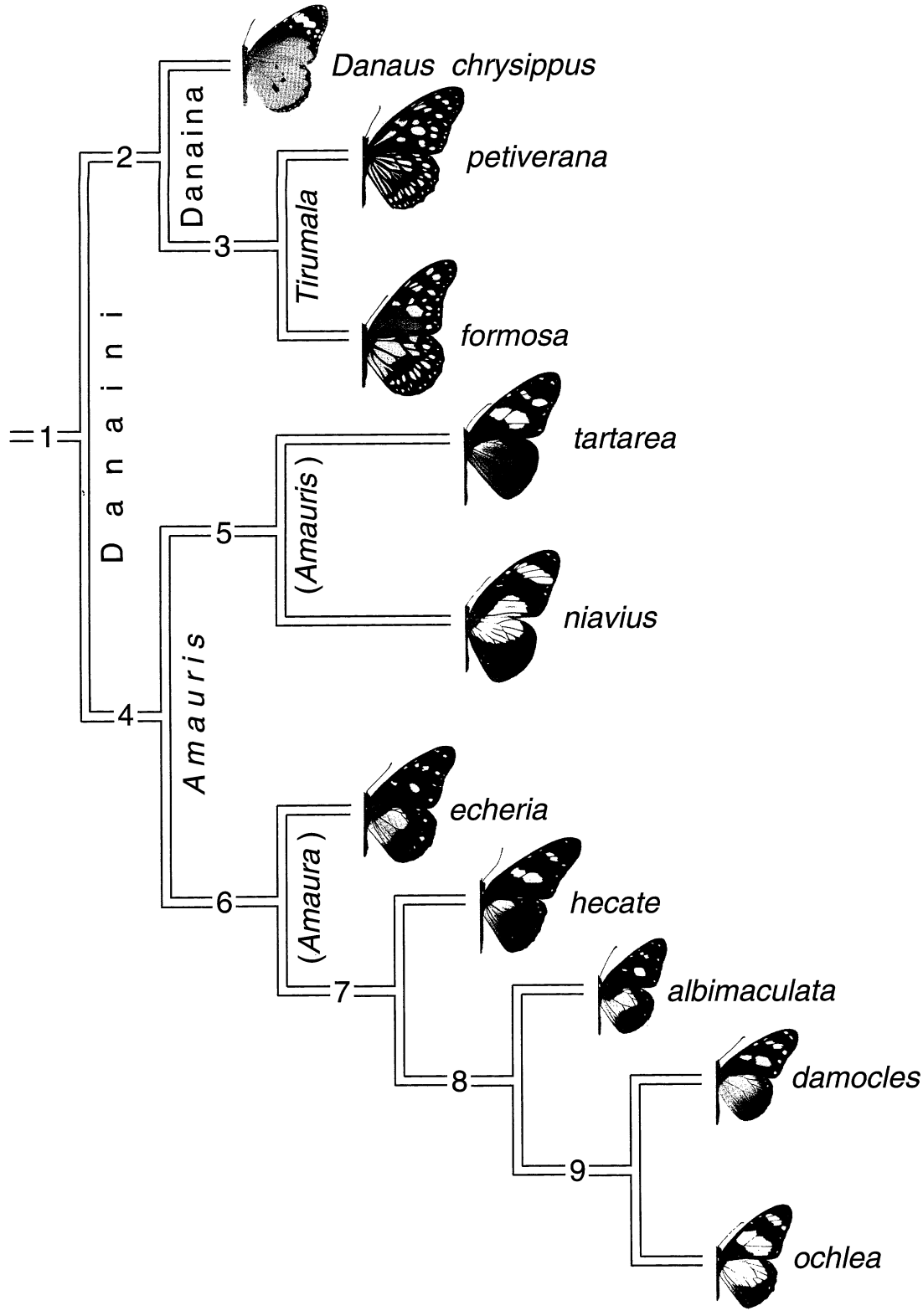

Figure 4. Cladistic relationships and generic groupings for the ten species of African milkweed butterflies. This 'standard tree' (Vane-Wright & Boppré 1993) is produced when either the 68 individual chemical characters (ch#, table 3; cf. Vane-Wright *et al.* 1992, table 3), or the 27 chemical groupings (table 6), are analysed in combination with the matrix for 32 morphological characters in Vane-Wright *et al.* (1992, table 2), using the HENNIG86 cladistics program (Farris 1988).

Table 6. *Chemical compounds grouped by established or assumed biosynthetic relationships*

(All data in table 3, except unknowns N1–16, hydrocarbons A13, A18 and A25 (*niavius* only), alcohols B1 and B2, lactone F3, oxidized carboxylic acids H1–H5 (*damocles* only), monoterpenes K1–K10, and terpenoids M1–M4 (*echeria* only) are summarized by this classification and the distribution matrix (1 = chemical group present; 0 = chemical group absent).)

straight-chain hydrocarbons
- group 1 alkenes shorter than tricosane (A1, A5, A7, A9, A11)
- group 2 alkanes shorter than tricosane (A2, A3, A4, A6, A8, A10)
- group 3 alkanes longer than docosane (A12, A14, A15, A16, A17, A22, A24)
- group 4 methyl-branched alkanes (A19, A20, A21, A23, A26, A27, A28)

aldehydes
- group 5 (all) (C1, C2, C3)

ketones
- group 6 even-numbered acyclic ketones (D1)
- group 7 odd-numbered acyclic ketones (D2–D10)
- group 8 cyclic ketones (D11)

esters
- group 9 (all) (E1–E3)

lactones
- group 10 C_9 lactones (F1, F2)
- group 11 C_{12} macrolides (F4, F5)
- group 12 C_{14} macrolides (F6, F7, F8)

carboxylic acids
- group 13 fatty acids shorter than tetradecanoic acid (G1–G13)
- group 14 fatty acids shorter than eicosanoic but longer than tridecanoic acid (G14–G32)
- group 15 fatty acids longer than nonadecanoic acid (G33–G43)

oxidized carboxylic acids
- group 16 keto and hydroxy fatty acids longer than undecanoic acid (H6–H17)
- group 17 saturated keto fatty acids longer than undecanoic acid (H6, H7, H9, H14, H15)
- group 18 unsaturated keto fatty acids longer than undecanoic acid (H8, H10, H11, H12, H13)
- group 19 hydroxy fatty acids longer than undecanoic acid (H16, H17)

aromatic compounds
- group 20 C_1 aromatic compounds (I1, I2, I9, I10)
- group 21 C_2 aromatic compounds (I3–I8)
- group 22 C_3 aromatic compounds (I11)

PA-derivatives
- group 23 (all) (J1–J3)

sesquiterpenes
- group 24 (all) (L1–L3)

other terpenoids
- group 25 C_{12} terpenoids (M5)
- group 26 C_{13} terpenoids (M6, M7, M8)
- group 27 C_{18} terpenoids (M9)

Danaus chrysippus	001 000 000 000 010 000 001 010 000
Tirumala petiverana	000 000 000 011 010 000 001 010 000
Tirumala formosa	001 000 001 000 010 000 000 000 000
Amauris tartarea	111 100 000 000 011 000 001 000 000
Amauris niavius	111 100 101 000 011 000 011 011 001
Amauris echeria	011 100 001 000 111 111 011 111 000
Amauris hecate	001 000 100 000 111 111 000 010 010
Amauris albimaculata	001 011 010 100 111 100 101 010 100
Amauris damocles	001 011 010 011 111 100 111 010 111
Amauris ochlea	001 011 010 110 111 100 110 111 000

both *niavius* and *tartarea*, the latter also including the respective phenol I7. *A. niavius* is particularly rich in hydrocarbons, including large amounts of relatively short-chained alkanes (group 2, starting with A3, octadecane), a group of compounds otherwise only found in *tartarea* and *echeria*. Other hydrocarbons, all unique to *niavius*, include A11, A13, A18, A21 and A25–A28. The only other compounds restricted to *niavius* are citral (K1 + K2), heneicosan-2-one (D10 – a compound related to the ketones of *hecate* – see below), and unknowns N9–N12. In addition, *niavius* exhibits relatively large amounts of aromatic compounds, one of the main components of the bouquet being methyl salicylate (I10), also present in *echeria* and *ochlea*, but only in minute amounts. In contrast, *tartarea* has only two unique compounds (A1, I7), of which only the hydrocarbon hexadecene occurs in quantity, showing a distinctive pattern more by absence than by presence. However, it would appear to be further differentiated by relative increase in four hydrocarbons: A14, A15, A20 and A27.

Subgenus *Amaura* (node 6) can be characterized by the presence of keto- and hydroxy-carboxylic (fatty) acids longer than undecanoic acid (group 16). In addition, carboxylic acids shorter than tetradecanoic acid (group 13) and docosanoic acid (G39) support this grouping within the African danaines. Oxidized carboxylic acids of groups 17 and 18 could also be considered characteristic of *Amaura*, if it is presumed that they have been lost secondarily in the *ochlea*-group. Of the species within subgenus *Amaura*, *echeria* branches off first (at node 6) and has a number of major compounds not found elsewhere, including large quantities of a unique blend of keto fatty acids (H7–H11, H13–H15), together with traces of the related methyl ester E3. Other unique compounds are the cyclic monoterpenes K3–K5 and the cyclic terpenoid ketones M1–M4. Aromatic compounds are particularly diverse in this species (I1, I4, I5, I10, I11), but only phenylacetaldehyde (I4) is a unique feature.

Node 7, subtending the last four *Amauris* species in the sample, is not well supported chemically. Only the carboxylic acid heptadecanoic acid provides a unique and un-reversed character at this point, and this substance has been found in non-African danaine species (S. Schulz, unpublished results). However, as already noted above, the loss of hydrocarbon groups 2 and 4 found in the three other *Amauris* species

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investigated provides some chemical support for this arrangement, as does the presence of lactones (this class is otherwise represented only in *petiverana*). The basal species at node 7, *hecate*, has two well-marked chemical autapomorphies: a unique series of methyl ketones (D2–D9) present in relatively large amounts, and the lactone 5-dodecanolide (F3). In addition, three of the group 16 keto fatty acids (H6, H9, H12) occur, representing a class of compounds also found in *echeria*, but only one compound (H9) is common to both species. In contrast, the related hydroxy acid H16 occurs solely in *hecate*, together with minor amounts of terpenoid M8.

The *ochlea* group (node 8: *albimaculata* and *ochlea*, plus the west African *damocles*) is the best chemically supported group in the set. All three species possess short-chain aldehydes (C1–C3; group 5), an even-numbered acyclic ketone (D1; group 6), a cyclic ketone (D11; group 8), some short-chain fatty acids (G2, G4, G5, G7), 13,16,19-docosatrienoic acid (G41), and 9-hydroxyoctadecanoic acid (H17), together with unknown compound N2.

Within the *ochlea* group of three species, two have marked autapomorphies: *albimaculata* is characterized by the short-chain hexanoic acid (G1), while *damocles* has the C_{13}-terpenoids dihydroedulan (M6, M7), the C_{22} fatty acids G42 and G43, the short-chain oxidized fatty acids H1–H5, and a high quantity of hydroxydanaidal (J3; but this PA-derivative recurs in *echeria*). *A. ochlea* lacks a major chemical autapomorphy (uniques F1, G32 and L2 are minor compounds) in relation to the allopatric *damocles*, but the two together (node 9) are characterized by several compounds which, as discussed under § 4*c*(i), could differentiate this species from *albimaculata* in east Africa: the C_{12} macrolide 11-dodecanolide (F4), and the relatively unusual (*Z*)-6-nonenoic (G6) and nonadecadienoic acids (G31).

With respect to resolution within the *ochlea*-group, according to the standard tree, the west/east African pair *damocles* and *ochlea* are sister species (node 9), excluding *albimaculata*. Grouping of *ochlea* and *damocles* is supported by the four compounds just discussed (F4, G6, G30, G31). However, some chemical characters are in conflict with this arrangement. Terpenoid M5 suggests that *albimaculata* rather than *ochlea* could be the sister of *damocles*, but this can be explained if we assume that presence of C_{12}-terpenoids (group 25) evolved with the *ochlea*-group (at node 7), but they have been lost subsequently in *ochlea* itself. Similarly, although the unsaturated acid G7 is uniquely present in all three *ochlea*-group species, the related aldehyde C3 occurs in *albimaculata* and *damocles* but not *ochlea*, whereas the respective lactone F2, absent in *damocles*, is present in *albimaculata* and *ochlea*, thus suggesting the third possible resolution (also supported by N1). It is this balance of four chemical characters supporting *ochlea* + *damocles*, in contrast to only two each supporting the two alternative arrangements, on which the resolution of node 9 in figure 4 depends.

Overall, we have a result in which the chemical data, in combination with the morphological data, recover the standard cladistic structure. The species of *Amauris* studied so far (7 out of 15) occur at four relative ranks, but there is no systematic increase or decrease in total number of individual compounds (although the least rich species, *tartarea*, occurs at the highest rank, while the richest, *ochlea*, occurs at the lowest, possibly suggestive of additivity). Thus we consider that the results favour both additive and subtractive modes, and Vane-Wright & Boppré (1993) have modelled such a process. There is little support for the idea of mixtures (although *tartarea* might be an exception, in relation to *niavius*), or that new species-specific compounds entirely replace or 'obliterate' previous stages in cladogenesis of the putative signal substances. However, there is some quantitative evidence that particular species-specific (autapomorphic) substances become major compounds within individual species, and could act as functional monospecific signal substances, as under the 'logos' hypothesis (Vane-Wright & Boppré 1993).

(*d*) *Mate recognition and mate choice in milkweed butterflies*

Chemical communication in the courtship of Danainae has been discussed elsewhere in some detail (Brower *et al.* 1965; Pliske & Eisner 1969; Boppré 1977, 1978, 1984, 1986, 1993; Eisner & Meinwald 1987; Schneider 1987). With the exception of *D. gilippus* (Brower *et al.* 1965; Pliske & Eisner 1969; Seibt *et al.* 1972), behavioural work has so far been mainly descriptive. A primary focus of interest has become the role of dihydropyrrolizine pheromone components (J1–J3): these compounds occur in almost all species, and often comprise the quantitatively dominant components of the hairpencil odour bouquets. In particular, their biosynthesis requires active collection of pyrrolizidine alkaloids by the adult males, usually from decaying parts of certain plants and independently of nutrient uptake. This fact is largely responsible for the great variation in the amount of PA-derived pheromones found in individual males. Another peculiar behaviour, performed separately from courtship activity, adds to this quantitative variation of dihydropyrrolizines: in addition to the hairpencils, males of most danaine genera possess glandular organs on the wings (cf. Boppré & Vane-Wright 1989), and – as has been shown for *D. chrysippus* (Boppré *et al.* 1978) – behaviourally mediated contacts between both glandular systems are necessary for production of normal amounts of danaidone (J1).

Dihydropyrrolizines, originally indentified in the danaine *Lycorea ceres* (Meinwald *et al.* 1966), gained additional interest when it was discovered that they also occur as male pheromone components in a variety of arctiid moths (see, for example, Culvenor & Edgar 1972; Conner *et al.* 1981; Schneider *et al.* 1982), as well as the closely related ithomiine butterflies (Schulz *et al.* 1988*b*). Courtship behaviour in these Lepidoptera differs markedly, however, and so the sexual or social messages that the dihydropyrrolizines convey in these diverse taxonomic groups are clearly not the same (for review and references, see Boppré (1986, 1990)).

Our understanding of PAs and PA-derived pheromones in danaines is greatly influenced by studies on

ithomiine butterflies and various moths; it is evident that PAs obtained from plants, in addition to being converted into pheromone components, are also stored by males in unconverted forms for chemical protection against predators. Furthermore, included with the male ejaculate, males donate PAs to females during copulation and, in turn, these protective chemicals are then incorporated into eggs (e.g. Brown 1984; Dussourd *et al.* 1989). It is therefore possible that, by perception of the dihydropyrrolizine pheromones, a female might assess how much PA she can expect to receive from a potential mate (cf. Eisner 1980; Conner *et al.* 1981; Eisner & Meinwald 1987; Dussourd *et al.* 1991).

Thus, although PA-derived pheromone components are likely to be involved in mate choice, because of their almost universal occurrence in the Danainae, these chemicals cannot mediate species recognition. A function of male pheromones in species recognition, however, must be expected and has been hypothesized because: (i) mate finding in butterflies is in general visually guided; and (ii) distinctive visual signs are lacking in milkweed butterflies, which are members of both Müllerian and Batesian mimicry rings, and thus tend to have very similar colour patterns which they also share with unrelated species (for review, see Vane-Wright & Boppré 1993). Previous chemical studies demonstrating the presence of non-dihydropyrrolizine volatiles in hairpencil extracts from a number of danaine species have failed to identify the chemicals (Edgar *et al.* 1973; Edgar & Culvenor 1974; Edgar 1975, 1982; Komae *et al.* 1982), but nevertheless support this view. The present study is the first systematic account of all volatiles encountered in the hairpencils of an entire community of danaines, and in which most of the chemical components have also been identified. Our results clearly indicate that the composition of hairpencil volatiles do have the potential to inform a female of the specific identity of a pursuer, because their bouquets are so distinct.

At this stage, it might be supposed that the successful identification of so many volatiles would now permit a series of behavioural tests. However, bioassaying courtship pheromones is far more complicated than, for example, testing trail or attractant pheromones, which usually cause very obvious and measurable changes in behaviour. Although for tests on male pheromones in moths one can take advantage of the females' overt demonstration of readiness to mate (luring behaviour), in butterflies, due to the crucial role of visual stimuli, behavioural tests of close-range chemical stimuli are far more complicated. These difficulties, together with the problem of obtaining sufficient numbers of adults in an appropriate physiological state for biotests, the longevity of danaines and, in particular, their frequent use of pheromone-transfer-particles (see below), frustrate almost any potential approach. Thus, faced with the complexity of their odour bouquets, at present we see no realistic prospect for conclusive experiments on the role(s) of male chemical stimuli in mate recognition among milkweed butterflies.

The precise roles of the various chemicals now identified must therefore remain a matter of speculation. However, considering the chemical results in relation to our understanding of the morphology and ecology of these butterflies permits us to make some further statements about the evolution of danaine signalling systems. Functionally, it seems certain that at least two types of information are transmitted from male to female by means of the androconial secretions. The pheromone bouquets have the capacity to convey information on both the species identity and the protective potency of individual males. In addition, certain components of the complex odours could serve as primer pheromones inducing, for example, oogenesis. This idea relates to the use of cuticular particles (cf. Boppré & Vane-Wright 1989) which, bearing pheromones (M. Boppré & S. Schulz, unpublished results), are applied from the hairpencils directly onto the female antennae during a final phase of courtship (Pliske & Eisner 1969; Boppré 1984). In consequence, the female olfactory receptors receive persistent stimulation, probably causing complete sensory adaptation. Furthermore, apart from transmitting signals, the use of pheromone-transfer-particles might also explain the males' extended hovering flights after visual approach to a female and prior to hairpencil expansion: the availability of particles in limited amounts seems to make males quite coy. However, experimental studies on the role(s) of pheromone-transfer-particles, which also occur in a variety of other Lepidoptera (Boppré 1994), are unlikely to be successful unless performed with species showing less complex behaviour patterns than the Danainae.

5. CONCLUSIONS

Our results provide further data on the complexity of male pheromone systems in milkweed butterflies, and also clearly demonstrate the potential value of small molecule studies for chemotaxonomy, including the reconstruction of phylogenetic relationships (cf. Morris & Cobabe 1991). However, we failed to obtain meaningful cladograms when trying to make a global analysis of similar chemical data for hairpencil extracts for all danaines so far investigated (Schulz 1977; Schulz *et al.* 1994), including non-African genera (*Lycorea*, *Euploea*, *Parantica*), together with American and Asian *Danaus*, and Asian *Tirumala* species. This result contradicts the value of such chemical data for cladistic studies only at first sight. There is no evidence to suggest that semiochemicals should reflect historical relationships of groups and species which have evolved in isolation.

In conclusion, we suggest that clear evidence of phylogenetic relationships from analysis of this kind of chemical data can only be expected when the species involved have evolved within a functional community, over an extended period of time. During evolution of aposematic species, particularly if mimicry is involved, chemical signals might become important to overcome problems of visual communication for mate location. In the most extreme case, i.e. during radiation to form monophyletic groups of co-mimics, stepwise evolution of chemical signals may be a necessary part of the

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speciation process (cf. Vane-Wright & Boppré 1993). Therefore, we suggest that only under a régime such as mimicry, which puts strong and continuing selective pressure on courtship signals, can we expect to find strong hierarchical patterns among scent organ chemicals, as in the *Amauris* species investigated here.

These hypotheses could be pursued in a wide range of taxa, including, for example, sympatric *Euploea* on different south-east Asian islands, or communities of South American Ithomiinae, the latter often involving a comparatively high number of genera and species (e.g. Papageorgis 1975). In this context, it will also be relevant to study further African danaine taxa, among which *Amauris nossima*, an endemic species of Madagascar, may be of special interest (Vane-Wright *et al.* 1992). According to morphological characters, *nossima* is the sister species of *ochlea* (Ackery & Vane-Wright 1984). In concordance with the data presented here, we could predict the hairpencil secretion of *nossima* males to show a very similar chemical profile to *ochlea*. However, we could also well imagine that this species has a quite different set of chemicals, simply because it appears to have evolved in complete isolation, separate and apart from all other *Amauris*.

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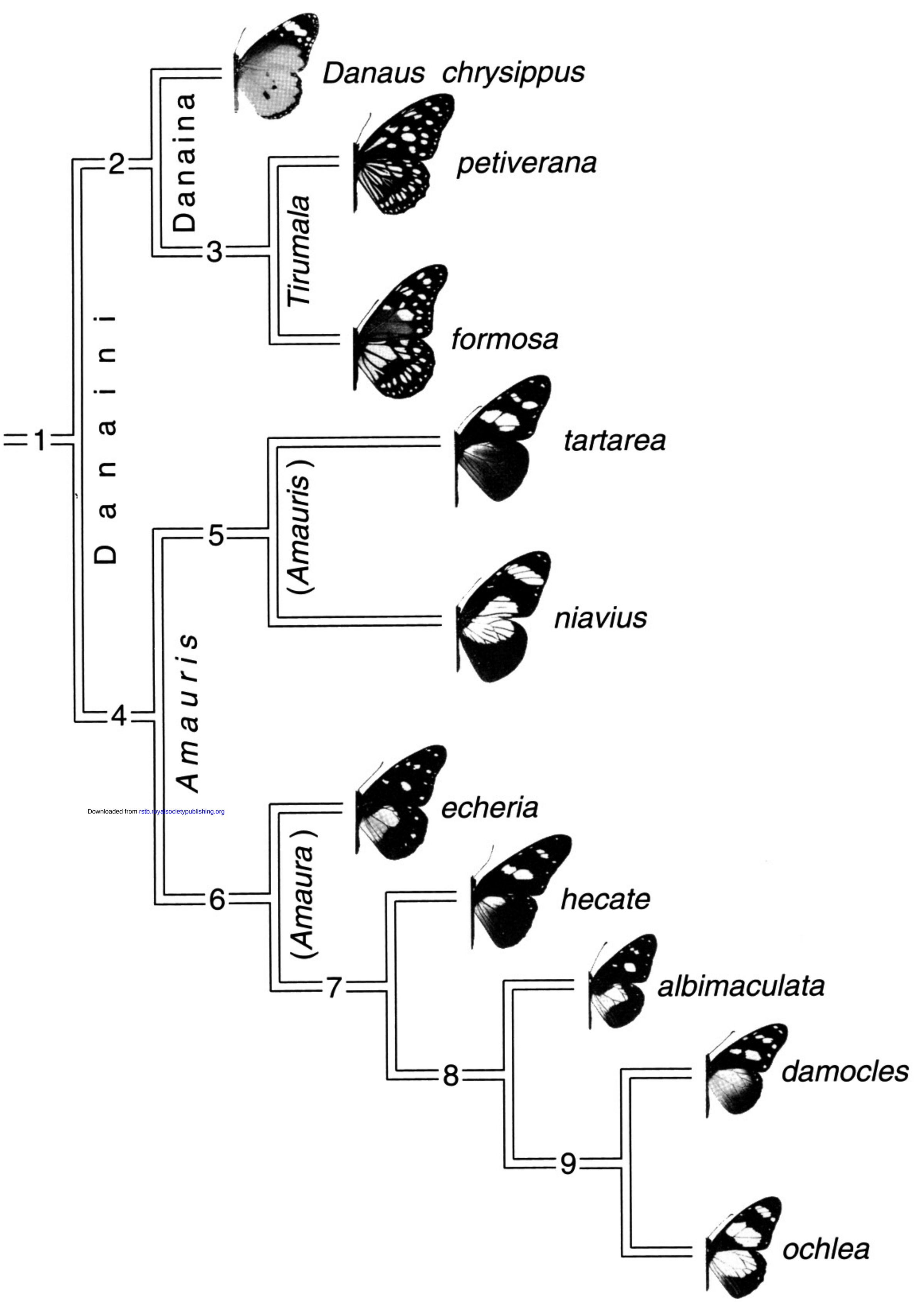

igure 4. Cladistic relationships and generic groupings for the ten species of African milkweed butterflies. This tandard tree' (Vane-Wright & Boppré 1993) is produced when either the 68 individual chemical characters (ch#, able 3; cf. Vane-Wright *et al.* 1992, table 3), or the 27 chemical groupings (table 6), are analysed in combination ith the matrix for 32 morphological characters in Vane-Wright *et al.* (1992, table 2), using the HENNIG86 ladistics program (Farris 1988).