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Biochemical Systematics and Ecology 32 (2004) 699–713

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Semiochemicals derived from pyrrolizidine alkaloids in male ithomiine butterflies (Lepidoptera: Nymphalidae: Ithomiinae)

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Received 5 June 2003; accepted 30 December 2003

Abstract

Analysis of male hairpencil components of 54 species in 30 genera of Ithomiinae butterflies showed the presence of 13 volatile compounds formed by hydrolysis, oxidation, lactonization and/or methylation of both necic acid and pyrrolizidine base portions of lycopsamine, an alkaloid widespread in nature and known to be specifically sought, ingested and stored by these insects. Six of these compounds have not been reported before from insects. Comparison of occurrence of components with proposed phylogenies of the 50 or more genera in the subfamily did not give clear patterns, though simpler derivatives well known in other Lepidoptera occurred throughout the subfamily, while more specific structures, including a lactone resulting from specific oxidation of an unactivated methyl group, were more typical of apomorphic genera.

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Keywords: Ithomiinae; Hydroxydanaidal; Methyl hydroxydanaidoate; Ithomiolide; Viridiflorin β -lactone; Male pheromones; Dihydropyrrolizines; Pyrrolizidine alkaloids; Alar fringes

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1. Introduction

Males of many Lepidoptera of the Arctiinae (tiger-moth), Danainae (monarchs), and Ithomiinae (glasswings) gather pyrrolizidine alkaloids (PAs), e.g. lycopsamine (**1**), pharmacophagously (i.e. for purposes other than nutrition) from certain types of dead or withering plant material or from flower sources and store these secondary metabolites for protection against predators. In addition, many use them as precursors for male pheromones (see e.g. Brown, 1984; Boppré, 1986; Eisner and Meinwald, 1987; Trigo et al., 1994). The basal ithomiine *Tithorea* and a close relative of the ithomiines, *Tellervo* (Tellervinae) have been shown to sequester PAs in the larval stage together with food (Trigo and Brown, 1990; Orr et al., 1996) and this is also true for numerous arctiines (Conner et al., 1981; Schneider et al., 1982; Dussourd et al., 1988, 1991; Krasnoff and Roelofs, 1989; Boppré and Schneider, 1989).

In the Arctiinae and Danainae, three major dihydropyrrolizine pheromone components are known to be synthesized from the necine base part of PAs. In the majority of species, hydroxydanaidal (**3**) is present; other often used compounds are danaidone (**2**) and danaidal (**4**) (Schulz, 1998; Hartmann and Ober, 2000). A derivative of the necic acid moiety of certain PAs, viridiflorine β -lactone (**12**), was found in these two families only in the danaine *Idea leuconoe* (Nishida et al., 1996; Schulz and Nishida, 1996; Stritzke et al., 2002). In ithomiines, however, in addition to **3**, the unique dihydropyrrolizine methyl hydroxydanaidoate (**5**) was reported (Schulz et al., 1988), as were the necic acid derived lactones ithomiolides A (**13**) and B (**14**) (Edgar et al., 1976; Schulz et al., 1988; Schulz, 1992). Lycopsamine (**1**) is the primary PA stored by Ithomiinae and it has so far been identified in 40 species from 38 genera (Trigo et al., 1996). It exhibits the same absolute configuration in its necic acid part as do the lactones **12** and **13** and it is, therefore, likely to be the direct biogenetic precursor of them. Intermedine, rinderine, and echinatine, diastereomers of **3**, are also gathered by adult Ithomiinae and are transformed by the butterflies into the putative pheromone precursor lycopsamine (Trigo et al., 1994).

Ithomiinae are interesting not only with respect to the evolution of use of PA derivatives in their chemical communication but also in terms of their sexual behavior. In danaines and many arctiines, androconial organs (abdominal hair brushes and coremata) emit short range courtship signals and are expanded after potential partners have met, whilst in some arctiine moths, these structures emit pheromones which function as long-range attractants, likely mediating the formation of leks (Willis and Birch, 1968; Wunderer et al., 1986). In contrast, Ithomiinae possess alar fringes rather than abdominal organs and these are sometimes displayed independent of the presence of females (see Section 4). In the present study, we report the analysis for PA derivatives in the alar fringes of 46 species and subspecies of Ithomiinae in 28 genera (over 300 species in 50 genera are known) and ask whether there is a phylogenetic pattern to their distribution.

2. Methods and materials

The butterflies were collected in the field in the 1980s, the beginning of the 1990s, and 2003. The scent glands were cut out and stored in dichloromethane or pentane until analysis. The individual samples contained between two and 30 butterflies. For each species, two to four samples were analyzed. These samples were investigated by gas chromatography and mass spectrometry on either a VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph, a Fisons MD-800 mass spectrometer coupled to a Fisons GC 8000, or a Hewlett-Packard HP 5973 MSD connected to a Hewlett-Packard HP 6890 gas chromatograph. FT-IR spectra were obtained by a Hewlett-Packard HP 5965A infrared detector connected to a Hewlett-Packard HP 6890 gas chromatograph. Different apolar phases like CP-Sil 8, DB-5 and BPX-5 were used for gas chromatography. Silylation was performed by adding 20 μ l MSTFA to about 20 μ l of an extract in a closed cap vial. After heating for 30 min at 50 °C, the solvent and excess MSTFA were removed with a gentle stream of nitrogen. The residue was taken up in CH_2Cl_2 and analyzed by GC-MS. Compounds **3–15** were identified by comparison of mass spectra and retention times with those of authentic reference samples.

2.1. *Hydroxydanaidal* (**3**)

EIMS (70 eV) 39 (21), 41 (7), 66 (15), 67 (9), 77 (14), 78 (12), 79 (18), 80 (17), 94 (52), 95 (61), 104 (26), 105 (21), 106 (18), 108 (10), 122 (36), 123 (63), 133 (16), 134 (32), 150 (27), 151 (100, M^+).

2.2. *Danaidal* (**4**)

EIMS (70 eV) 50 (12), 51 (19), 52 (10), 63 (7), 77 (26), 78 (17), 79 (27), 104 (29), 105 (10), 106 (67), 133 (10), 134 (88), 135 (100, M^+).

2.3. *Methyl hydroxydanaidoate* (**5**)

EIMS (70 eV) 39 (17), 51 (11), 65 (10), 66 (11), 67 (5), 78 (10), 79 (12), 93 (45), 94 (22), 104 (30), 105 (21), 120 (27), 121 (41), 122 (53), 125 (22), 132 (21), 148 (70), 149 (23), 150 (41), 153 (60), 164 (42), 180 (11), 181 (100, M^+).

2.4. *Methyl oxodanaidoate* (**6**)

EIMS (70 eV) 64 (23), 65 (13), 93 (57), 120 (64), 121 (23), 148 (100), 149 (31), 164 (6), 179 (68, M^+).

2.5. *Oxodanaidal* (**7**)

EIMS (70 eV) 38 (16), 39 (15), 64 (25), 65 (26), 66 (19), 93 (66), 94 (14), 120 (61), 121 (94), 148 (11), 149 (100, M^+).

2.6. *Loroquine* (**8**)

EIMS (70 eV), 39 (27), 41 (10), 51 (15), 52 (17), 65 (10), 66 (20), 67 (24), 68 (12), 78 (13), 80 (22), 91 (11), 94 (23), 95 (14), 106 (27), 108 (20), 120 (10), 122 (100), 134 (8), 151 (71, M⁺); Trimethylsilyl ether: EIMS (70 eV), 45 (7), 51 (8), 73 (12), 78 (10), 106 (27), 134 (72), 208 (100), 223 (6, M⁺).

2.7. *9-O-Methyl-hydroxydanaïdol* (**9**)

EIMS (70 eV) 39 (11), 45 (5), 51 (14), 52 (15), 53 (15), 78 (11), 79 (22), 80 (15), 93 (10), 94 (52), 106 (31), 107 (21), 108 (11), 117 (12), 118 (25), 119 (14), 120 (18), 134 (18), 135 (13), 136 (100), 137 (16), 149 (4), 150 (9), 166 (12), 167 (62, M⁺); Trimethylsilyl ether: EIMS (70 eV) 45 (19), 73 (42), 75 (11), 91 (11), 104 (10), 105 (10), 106 (17), 117 (18), 118 (50), 119 (23), 120 (74), 134 (47), 149 (13), 150 (39), 166 (10), 194 (34), 208 (100), 209 (31), 224 (11), 239 (58, M⁺).

2.8. *9-Methoxydanaïdon* (**10**)

EIMS (70 eV) 39 (8), 45 (3), 51 (22), 52 (16), 65 (5), 66 (5), 78 (20), 79 (13), 80 (16), 93 (3), 94 (3), 106 (70), 107 (20), 108 (7), 122 (7), 134 (55), 135 (100), 136 (18), 150 (9), 164 (6), 165 (8, M⁺); FT-IR 2914 (m), 2832 (w), 1728 (s), 1387 (m), 1201 (m), 1109 (m), 739 (m).

2.9. *9-O-Methyl-retronecin* (**11**)

EIMS (70 eV), 39 (10), 45 (17), 53 (15), 67 (13), 79 (16), 80 (100), 93 (42), 94 (39), 95 (18), 110 (11), 125 (28), 138 (24), 139 (13), 149 (2), 169 (19, M⁺); Trimethylsilyl ether: EIMS (70 eV), 45 (30), 59 (13), 73 (24), 80 (67), 93 (46), 94 (65), 95 (33), 110 (11), 120 (12), 125 (100), 241 (15, M⁺).

2.10. *Viridiflorine β-lactone* (**12**)

EIMS (70 eV) 39 (26), 41 (33), 43 (100), 44 (8), 45 (44), 55 (17), 56 (6), 57 (77), 67 (22), 71 (30), 72 (16), 73 (7), 85 (39), 100 (30, M⁺–44).

2.11. *Ithomiolide A* (**13**)

EIMS (70 eV) 39 (12), 41 (20), 42 (20), 43 (26), 44 (3), 45 (38), 55 (53), 56 (17), 57 (9), 70 (46), 71 (22), 101 (32), 116 (100, M⁺–44).

2.12. *Ithomiolide B* (**14**)

EIMS (70 eV) 39 (11), 41 (20), 42 (13), 43 (100), 44 (8), 45 (12), 55 (25), 57 (14), 69 (6), 70 (24), 71 (20), 72 (10), 101 (21), 116 (71, M⁺–42).

2.13. *1'-O-Acetylithomiolide A* (**15**)

EIMS (70 eV) 39 (6), 41 (10), 42 (8), 43 (100), 45 (8), 55 (11), 56 (21), 70 (15), 71 (8), 83 (6), 87 (8), 98 (10), 101 (8), 102 (5), 116 (87), 158 (7, M⁺–44).

The experiments comply with the current laws of the countries in which they were performed.

3. Results

The alar fringes of butterflies collected in the field were excised, stored in CH_2Cl_2 and analyzed by GC–MS. The results are compiled in Table 1, together with earlier results from Edgar et al. (1976) and from us (Schulz et al., 1988). Fifteen and 12 species contained the necine base derivatives **3** and **5**, respectively, while 18 were found to possess the lactone **13**. Some species contained rarer PA derivatives. In *Melinaea menophilus* and *Scada kusa*, viridiflorine β -lactone (**12**) was found, a known pheromone of the danaine *I. leuconoe* (Nishida et al., 1996; Schulz and Nishida, 1996). Biosynthetically, this lactone can easily be formed by simple internal ring closing esterification of the necic acid part of **1**. *Mechanitis polymnia* contains a diverse array of dihydropyrrolizines. Besides the major component hydroxydanaidal (**3**), minor amounts of **5** and its oxidation product methyl oxodanaidoate (**6**) were found. Additionally, traces of oxodanaidal (**7**) occur. Compounds **6** and **7** have not been found before in nature. Their structures were derived from their mass spectra and confirmed by comparison with synthetic samples. While **7** occurs in traces in two other species, the known danaidal (**4**), a compound typical for danaines, was found only in *Episcada carcinia*. Four unique dihydropyrrolizine derivatives occur in the alar fringes of *Napeogenes sylphis caucayaensis*. Trace amounts of loroquine (**8**), which has been previously isolated from an Apocynaceae plant (del Castillo et al., 1970), were detected by comparison with a synthetic sample. The three other components are all O-methylated at C-9, indicated by the presence of an ion at $m/z = 45$ in their mass spectra, which corresponds to a $[\text{CH}_3\text{O-CH}_2]^+$ fragment. Furthermore, two compounds were silylated after treatment with trimethylsilyltrifluoroacetamide (MSTFA), indicating the presence of a hydroxy group. The corresponding mass spectra also did not show an ion at $m/z = 103$ $[(\text{CH}_3)_3\text{Si-O-CH}_2]^+$, typical for the presence of a hydroxymethyl group in dihydropyrrolizines. One component also exhibited a loss of 44 amu from the molecular ion (equivalent to 116 amu after treatment with MSTFA), which can be explained by a removal of C-6 and C-7 from a dehydropyrrolizidine nucleus (Neuner-Jehle et al., 1965). The FT-IR spectrum of one compound indicated the presence of a keto group. After comparison with synthetic samples, the three compounds were identified as 9-O-methyl-hydroxydanaidol (**9**), 9-methoxydanaidone (**10**), and 9-O-methyl-retronecine (**11**). They have also not been reported from nature before. Furthermore, the unique methyl ether function is not present in other dihydropyrrolizines from Lepidoptera. It is well known that hydroxydanaidol, an intermediate in the biosynthesis of hydroxydanaidal (Schulz et al., 1993), is unstable and reacts readily with methanol to form ethers like **10**. Nevertheless, no methanol containing solvent was used and other species collected during the same field work contained no trace of these compounds, while they were consistently present in different *N. sylphis caucayaensis* samples, thus proving their natural origin.

Table 1 (continued)

	Country	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>Tithorea harmonia furid</i> ^f	Venezuela			++										
<i>Tithorea harmonia pseudethra</i>	Brazil			++										
<i>Tithorea harmonia hermitas</i>	Ecuador													

+++ , major component; ++ , minor component; + , trace component.

^a Data taken from the literature (Edgar et al., 1976).

^b Originally described as *Hymenitis* by Edgar et al. (1976).

^c Laboratory culture with access to different PA sources, including plants containing lycopsamine type alkaloids.

^d According to G. Lamas (in literature) belonging to a gen. Nov.

^e Considered as sp. of *Greta* by K.S. Brown (unpublished).

^f Data taken from the literature (Schulz et al., 1988).

^g Probably conspecific with *M. polymnia*.

^h Data taken from the literature (Schulz, 1992).

Another new compound occurring in traces accompanying **13** is 1'-*O*-acetylthiomolide A (**15**), which was identified in *Ceratinia tutia* and *Pteronymia vestilla*. Generally, **3**, **5**, and **9–14** are prominent components of the androconia, while all other compounds occur only as minor or trace compounds along with them. Often the PA derivatives are accompanied by complex mixtures of other, non-PA derived, components (Schulz et al., 1988), which might serve as lipidic matrix for the more polar semiochemicals. The identification of these will be reported elsewhere. Only three species, *Episcada clausina*, *M. menophilus*, and *Prittwitzia hymenaea* contained necic acid and necic base derived compounds at the same time. In *Hypothyris*, both types also occur, but in different species.

Twenty species did not contain any alkaloid derivative. Probably, the specimens investigated did not ingest any, because plants containing them were not available at the time, and collected individuals might not have had access to them. Not all samples of a given species contained PA derivatives, supporting this explanation. Also, in some genera only one species contained these compounds, while a congeneric one did not (see e.g. *Ithomia agnosia* and *I. salapia*; *Hypoleria arzalia* and *H. orolina* versus *H. proxima*). Nevertheless, it cannot be completely ruled out that these species do not use any PA for semiochemical formation. For example, all the *Oleria* samples collected at different times in different locations never contained any PA derivatives. In *Dircenna dero*, only one of six samples contained traces of **3**.

The pattern of PA use for pheromone formation is obviously diverse. Some species gather PAs but may not use them for synthesizing pheromone components; others simply use the only slightly modified β -lactone **12**; yet others produce one or several dihydropyrrolizines from the necine base part of PAs and also lactones derived from a necic acid.

4. Discussion

The biosynthetic pathways to these semiochemicals has so far only been elucidated for **3**, which has been shown to be derived from PA in an arctiid moth (Bell et al., 1984; Schulz et al., 1993). It includes formation of the pyrrole ring, followed by ester cleavage which removes the necic acid, and finally oxidation to the aldehyde. Further oxidation and methylation leads to the ester **5**. Oxidation of the secondary alcohol present in both **3** and **5** leads to the keto compounds **6** and **7**. Interestingly, **8** was found as a by-product during the biosynthetic studies of **3**, but is itself not transformed by the moth or stored in the androconia (Schulz et al., 1993). The compounds **9–11** follow the same oxidation patterns with different regioselectivity. The only differences seem to be that the C-9 hydroxyl group is obviously methylated before further oxidations occur, because the derivative **11** is present. So far, it is the only necine derivative found in androconia of Lepidoptera which contains no pyrrole ring.

The biosynthesis of the lactones is closely related to viridifloric acid, the necic acid part of **1** (as noted above). The β -lactone **12**, albeit unusual, is relatively easily

formed by simple enzyme mediated esterification of the hydroxy and acid groups already present in viridifloric acid.

The biosynthesis of the lactones **13–15** requires that the butterflies possess an enzyme which oxidizes one of the unactivated prochiral methyl groups of the isopropyl side chain of viridifloric acid. The resulting hydroxy acid can then easily be esterified by an esterifying enzyme to form the γ -lactone ring.

The semiochemicals identified in the present study can thus be divided into three classes: (1) necine base derived dihydropyrrolizines, which differ only in the oxidation status (**3–11**); (2) relatively unmodified necic acid derivatives with no change of oxidation status like **12**; (3) uniquely modified necic acid derivatives (**13–15**). The structures and major biosynthetic manipulations are shown in Fig. 1.

PAs are unstable compounds, which degrade slowly when exposed to the environment, for example in withering plants. Among the degradation products of PAs, **3** has been detected (Schulz and Boppré, unpublished results), which can be produced by cleavage of the alkaloid ester bonds and oxidation. It has also been found in extracts of withering parts of *Heliotropium amplexicaule* by Edgar et al. (1973). The fact that **3** is not only produced by many butterflies and moths, but is

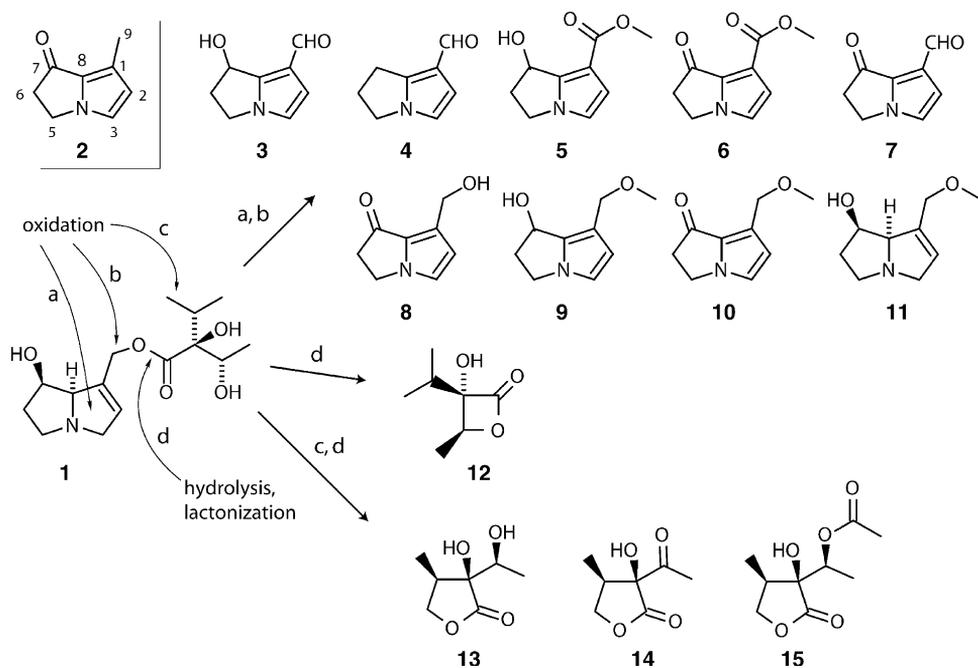


Fig. 1. Conversion of pyrrrolizidine alkaloids to volatile dihydropyrrolizines or necic acid lactones by male ithomiine butterflies. Compound **2** occurs only in danaines. Relative configurations for **11–14**; only in *Prittwitzia hymenaea* was the absolute configuration of **13** and **14** determined to be that shown (Schulz, 1992), while in the danaine *Idea leuconoe*, **12** has the shown absolute configuration (Stritzke et al., 2002).

also released by decaying plant material, makes it unlikely that butterflies are using this compound for species discrimination. It is very likely that the other group 1 compounds are also formed during the degradation process of PAs under natural conditions. The only group 2 compound, **12**, may also be formed from degrading PAs from the necic acid part under natural conditions, although the formation of a strained β -lactone is reasonably difficult and we have not identified it in decaying plants so far. Nevertheless, [Pliske et al. \(1976\)](#) have shown that adult ithomiines are attracted by degrading necic acid.

In contrast to groups 1 and 2, the lactones **13–15** cannot easily be formed by degradation, because they require the oxidation of a non-activated methyl group without affecting other parts of the molecule, which is not likely to occur under exposure to air, radiation and humidity. While dihydropyrrolizines have been detected in many lepidopteran genera ([Schulz, 1998](#)), and **12** has been found in a danaine butterfly (see above), isopropyl oxidation of viridifloric acid seems to be restricted to ithomiines.

In order to investigate whether there is any phylogenetic pattern in the distribution of PA derivatives in the Ithomiinae, we recorded the presence or absence of the compounds listed in [Table 1](#) on cladograms of ithomiine taxa published by [Brown and Freitas \(1994\)](#) and [Brower \(2000\)](#) ([Figs. 2 and 3](#), respectively). Whilst group 1 type semiochemicals are scattered across both trees and the group 2 lactone occurs only twice, group 3 lactones are not found in taxa belonging to the tribes Mechanitini, Melinaeini and Tithoreini. These tribes are believed to be basal to the rest of the Ithomiinae ([Fox, 1949](#); [Brown and Henriques, 1991](#); [Brower, 2000](#)) which, if correct, suggests that the ability to synthesize the more complex γ -lactones evolved in the common ancestor of the more highly derived tribes. Additional work on the phylogenetic relationships of the Ithomiinae and more data on the distribution of PA derivatives in ithomiine taxa is needed, however, before this can be accepted.

We would not be surprised, if future studies find no further clear phylogenetic patterns in the distribution of PA derivatives in the Ithomiinae, since the evolution of compounds used in pheromone communication may be governed more by conditions of the biotic environment than by phylogenetic relationships. Thus, pheromone chemistry (and androconial morphology, too) may not necessarily be strongly correlated with the species' phylogeny, particularly given a system so deeply involved in highly complex mimicry associations as are the ithomiines ([Beccaloni, 1997](#)). Ithomiinae are clearly different to danaines and arctiines through their ability to synthesize group 3 type γ -lactones.

A functional explanation requires further behavioral data, since the function of the alar fringes is still open to debate. In some arctiines and danaines **1** and **3**, have been shown to act as courtship pheromone at close range ([Pliske and Eisner, 1969](#); [Krasnoff and Dussourd, 1989](#)). In *Utetheisa ornatrix*, the amount of pheromone is correlated to the amount of PA transferred during copulation to the female ([Dussourd et al., 1988](#)); attraction of females to males from some distance has also been observed in *Cretonotos transiens* ([Boppré and Schneider, 1989](#)) and *I. leuco-noe* ([Nishida et al., 1996](#)).

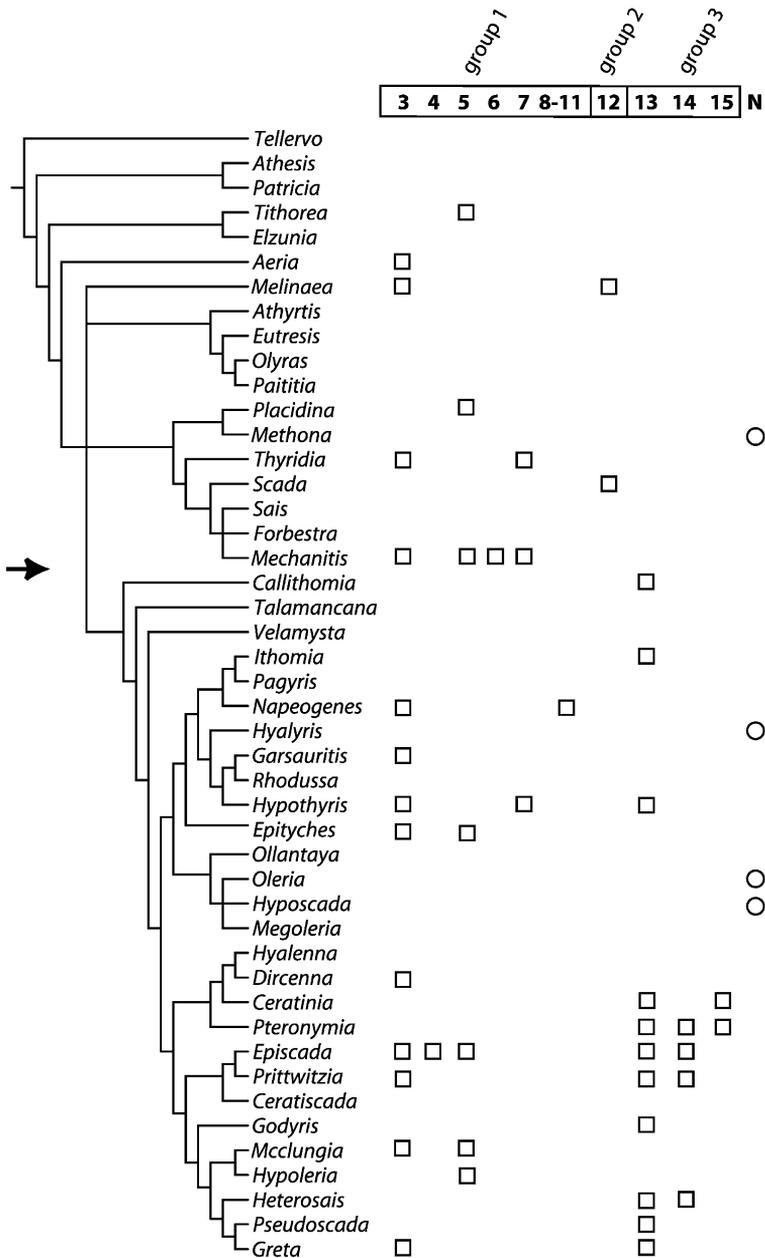


Fig. 2. Cladogram of Ithomiinae genera based on total evidence after Brown and Freitas (1994). The tribes Mechanitini, Melinaeini, and Tithoreini are above the arrow. Presence of volatile alkaloid derivatives is denoted by a box under the respective compound number. Genera in which species analyzed contained no PA derivatives are indicated under N with a circle.

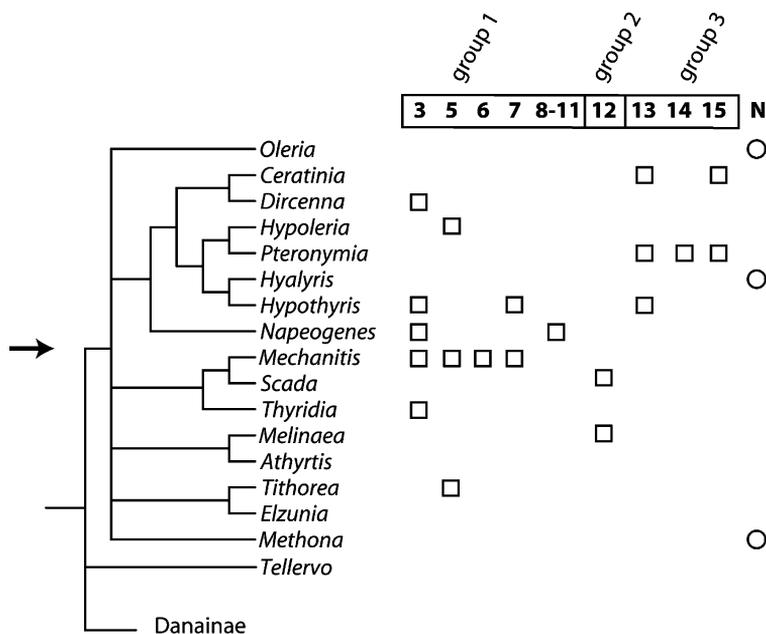


Fig. 3. Cladogram of Ithomiinae genera based on total evidence after Brower (2000) (order of the taxa reversed to enable easier comparison with Fig. 2). The tribes Mechanitini, Melinaeini, and Tithoreini are above the arrow. Presence of volatile alkaloid derivatives is denoted by a box under the respective compound number. Genera in which species analyzed contained no PA derivatives are indicated under N with a circle.

Whether these mechanisms are also acting in ithomiines is unknown. Males of many, but not all, ithomiine species, often perch in a particular posture with these organs erected, inside the forest in particular areas of aggregation. Haber (1978) showed that these organs attract both male and female ithomiines to aggregations or leks with up to 30 species which may last for weeks. Edgar et al. (1976), in contrast, postulated male–male repellency and territorial marking as functions of the emitted chemicals. In both these scenarios, the pheromones need not to be species-specific, which is in accordance with the pattern of occurrence of PA derivatives we report. Additional non-PA-derived compounds of which many already have been found and analyzed (Schulz and Beccaloni as well as Schulz, Brown, and Trigo, unpublished) may be used for species recognition prior to mating. It would be interesting to investigate whether there is a relationship between the production of group 3 lactones and the formation of leks.

Acknowledgements

We would like to thank D. Schneider for providing samples and the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie for financial

support. Work in Brazil was supported by the CNPq and FAPESP (Grants 98/05101-8 and 00/01484-1), as well as CAPES/DAAD (to J.R.T.). We also thank T. Hartmann, Braunschweig, for helpful remarks on the manuscript.

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