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DEFENSIVE CHEMICALS OF TWO SPECIES OF *Trachypachus* MOTSCHULSKI

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Abstract—Analyses of pygidial gland contents of two species of a previously uninvestigated family of beetles (Trachypachidae) by Gas Chromatography-Mass Spectrometry (GC-MS) revealed that their chemistry is similar to that reported from many members of the family Carabidae. Nevertheless, the composition of defensive gland fluids of the two species *Trachypachus slevini* and *T. gibbsii* differs sufficiently to distinguish between the two species solely on the basis of their defensive chemistry. The major components of *T. slevini* glandular fluid are methacrylic, tiglic, and octanoic (= caprylic) acids, together with the hydrocarbon (*Z*)-9-pentacosene. In contrast, the glandular contents of *T. gibbsii* contain a rather unique mixture of polar and nonpolar compounds, the principal constituents of which are methacrylic and ethacrylic acids (= 2-ethylacrylic acid), together with 2-phenylethanol, 2-phenylethyl methacrylate, 2-phenylethyl ethacrylate, and (*Z*)-9-pentacosene.

Key Words—Defensive secretion, methacrylic acid, tiglic acid, octanoic acid, Trachypachidae, *Trachypachus slevini*, *Trachypachus gibbsii*.

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INTRODUCTION

The extraordinary repertory of defensive chemicals used by beetles has undoubtedly contributed to their widespread success in terrestrial ecosystems (Meinwald and Eisner, 1995). Adephaga, the second largest suborder of beetles (Coleoptera), comprising approximately 40,000 species, is the best investigated group both at the species level and across families. Chemical studies on more than 500 species of Adephaga show that their secretions are rather complex mixtures of polar and nonpolar compounds (Blum, 1981; Dettner, 1987; Will et al., 2000). We recently studied two species of the previously uninvestigated adephagan beetle family Trachypachidae. We report here on the chemical composition of the pygidial gland defensive fluid of *Trachypachus slevini* Van Dyke and *Trachypachus gibbsii* LeConte, suggesting that both species have chemical defensive strategies similar to those that have been used by another adephagan family, Carabidae. This implies a possible close relationship between the two families.

Trachypachidae is one of the smallest families of Coleoptera with only two genera, *Trachypachus* Motschulski and *Systolosoma* Solier. The family's amphitropical distributional pattern is disjunct and relictual. The four species of *Trachypachus* are distributed in western Nearctic (three species) and eastern Palearctic (one species) regions. *Systolosoma* has two described species restricted to Chile and Argentina.

The monophyly of Trachypachidae and its membership in the suborder Adephaga are well supported (Beutel, 1995). Its relationship to other adephagan families, however, is contentious. Some analyses place them as a sister group of Carabidae (e.g., Kavanaugh, 1986; Shull et al., 2001). Other authors, on the basis of various character systems, place them as a sister group to all or part of the aquatic taxa in Hydradephaga (Bell, 1966; Arndt, 1993; Deuve, 1993; Beutel and Haas, 2000). Although a variety of morphological characters have been studied, the hypothesized direction of character change and significance have been challenged (Kavanaugh, 1986). To date, only a single gene sequence, using one species from each trachypachid genus, has been investigated for familial level relationships in Adephaga (18S ribosomal RNA, Shull et al., 2001).

Pygidial gland secretions are ecologically and phylogenetically significant (Moore and Brown, 1979; Dettner, 1985, 1987; Will, 2000; Will et al., 2000). With this in mind, we have identified the volatile constituents of pygidial glands in *T. slevini* and *T. gibbsii* and have interpreted them in terms of phylogeny and ecology. A number of compounds that we identified from these beetles are significant as secondary metabolites because of their scattered and rare occurrence in arthropods.

METHODS AND MATERIALS

Insects. Live beetles were collected in California, Oregon, and Washington states and transported to laboratory facilities in Berkeley, California, and Tucson,

Arizona. Beetles were kept cool and fed both chopped mealworm larvae (*Tenebrio*) and commercial dog food. Dissection and secretion sampling methods followed Will et al. (2000). Sealed glass ampoules containing excised gland reservoirs were shipped to laboratory facilities in Ithaca, New York or Hoboken, New Jersey for chemical analysis. Ampoules containing the gland reservoirs were kept frozen until chemical analysis could be performed.

Reference Compounds and General Procedures. Ethacrylic acid, isopropyl methacrylate, isopropyl ethacrylate, 2-phenylethyl methacrylate, and 2-phenylethyl ethacrylate were synthesized. Other reagents were available in our collection of chemicals, or purchased from Aldrich Chemical Company. Synthetic reactions were monitored by TLC. Electron-ionization (EI) mass spectra were measured by GC-MS using an HP 5890 II gas chromatograph (GC) linked to an HP 5989A mass spectrometer. Infrared spectra of synthetic compounds were measured on a Perkin–Elmer Paragon 1000 PC Fourier Transform Infrared (FT-IR) spectrometer.

Analytical Procedures. For gas chromatographic analysis of glandular extracts of *T. slevini*, the tubes were opened, and each sample was extracted with 20 μl of dichloromethane or hexane, and a 0.5 μl aliquot of each sample was analyzed by GC-MS using an HP 5890 GC linked to an HP 5970 mass selective detector [25 m $\times$ 0.22 mm fused-silica column coated with HP-1; the oven temperature was kept at 60°C for 4 min and raised 10°C/min to 260°C], or a Micromass Autospec mass spectrometer [Analyses were performed using a 25 m $\times$ 0.25 mm fused-silica column coated with 0.25 μm ZB-FFAP (nitroterephthalic acid modified polyethylene glycol; Phenomenex, Torrance, CA)]. The oven temperature was kept at 40°C for 3 min and raised 6°C/min to 265°C].

Gland reservoir contents of *T. gibbsii* were extracted with hexane (20 μl) and 1 μl of the extract was injected into the GC-MS [HP 5989A mass spectrometer linked to an HP 5890 II GC equipped with a ZB-FFAP-coated (0.25 μm) fused-silica capillary column (30 m $\times$ 0.25 mm)]. The oven temperature was held at 30°C for 2 min and increased at 6°C/min to 240°C.

GC-FTIR analyses were performed using an Infrared Detective Instrument (Bourne Scientific, Acton, MA) linked to a Shimadzu 17A GC. The GC was fitted with a 15 m $\times$ 0.25 mm fused-silica column coated with RTX5 (0.25 μm). Compounds eluting from the GC column were passed through a transfer line (300°C) and frozen on a rotating ZnSe window (speed: 2 mm/min) at -65°C, and transmission FT-IR spectra were recorded.

*Synthesis of Ethacrylic Acid (= 2-Ethylacrylic Acid) (**3**).* Ethyl 2-ethylacrylate (**2**): A solution of triethyl phosphonoacetate (**1**, 0.341 ml, 1.68 mmol; 98% purity) in tetrahydrofuran (0.5 ml) was added dropwise to a suspension of NaH (67.4 mg, 1.69 mmol) in mineral oil (60%) at 0°C. The reaction mixture was stirred at room temperature for 0.5 hr, cooled to 0°C, and EtI (0.136 ml, 1.69 mmol) was added dropwise and stirred at room temperature for 14 hr. A further aliquot of NaH (67.4 mg, 1.69 mmol, 60% in mineral oil) was added at 0°C, and the reaction mixture was stirred at room temperature (1 hr). Paraformaldehyde (58.6 mg,

1.85 mmol, 95% purity) was added at room temperature, and the mixture was stirred for 1 hr. After diluting with H_2O (1 ml), the mixture was extracted with pentane (3×0.5 ml). Combined pentane extract was washed with H_2O (2×0.5 ml) and evaporated to about 0.25 ml and used in the next step. Ethyl 2-ethylacrylate (**2**): EIMS m/z: 128[M]$^+$ (8), 113 (19), 100 (18), 95 (3), 85 (13), 83 (52), 82 (29), 69 (3), 55 (100), 45 (18), 43 (9), 39 (22).

*2-Ethylacrylic Acid (**3**)*: A mixture of **2**, NaOH (75 mg, 1.875 mmol), MeOH (0.5 ml), and H_2O (0.5 ml) was stirred on an oil bath at 100°C for 4.5 hr. Methanol was evaporated, and the mixture was acidified (Conc. H_2SO_4 0.1 ml + 0.5 ml water), and saturated with NaCl. The product was extracted into CH_2Cl_2/pentane (1:1; 3×0.5 ml) and dried over $MgSO_4$. The solvent was evaporated to give a colorless liquid **3** (102 mg, 59% total yield). IR (neat) ν_{max} 2975, 1698, 1629, 1446, 1194 cm^{-1}; EIMS m/z: 100 [M]$^+$ (47), 99 (4), 85 (29), 82 (25), 73 (7), 72 (9), 60 (5), 55 (100), 50 (11), 45 (23), 44 (24), 39 (48).

Synthesis of 2-Phenylethyl and Isopropyl Esters. The acid (6 mg, methacrylic or ethacrylic) was mixed with 2-phenylethyl or isopropyl alcohol (1 eq.), dicyclohexylcarbodiimide (1 eq.), and a catalytic amount of 4-dimethylaminopyridine in dichloromethane. The mixture was stirred overnight and flashed through a silica gel column with hexane:diethyl ether (6:4). *Isopropyl methacrylate*: EIMS m/z: 128 [M]$^+$ (4), 113 (8), 88 (2), 87 (54), 86 (5), 83 (7), 82 (3), 74 (2), 70 (13), 69 (99), 59 (28), 58 (2), 45 (7), 44 (2), 43 (84), 42 (18), 41 (100), 39 (51), 38 (6), 37 (3). *Isopropyl ethacrylate*: EIMS m/z: 142[M]$^+$ (2), 109 (2), 102 (2), 101 (33), 104 (44), 97 (2), 85 (14), 84 (9), 83 (78), 82 (17), 81 (2), 73 (2), 69 (4), 59 (21), 58 (2), 57 (7), 56 (10), 55 (84), 54 (11), 53 (11), 52 (2), 51 (3), 50 (2), 45 (6), 44 (4), 43 (100), 42 (13), 41 (39), 39 (33), 38 (3). *2-Phenylethyl methacrylate*: EIMS m/z: 105 (11), 104 (100), 103 (5), 91 (8), 79 (3), 78 (5), 77(5), 69 (32), 65 (5), 63 (2), 51 (4), 41 (33), 40 (3), 39 (13). *2-Phenylethyl ethacrylate*: EIMS m/z: 105 (14), 104 (100), 103 (5), 91 (6), 83 (16), 79 (3), 78 (5), 77 (6), 65 (4), 56 (2), 55 (31), 54 (2), 53 (4), 51 (4), 39 (9).

Determination of Position of Double Bonds. Glandular extracts were methylthiolated according to Francis and Veland (1981). The adducts were extracted into dichloromethane and subjected to GC-MS analysis [Shimadzu GCMS-QP5050A fitted with an XTI-5-coated (0.25 μm) fused-silica capillary column (30 m $\times$ 0.25 mm)]. The oven temperature was held at 40°C for 3 min, increased at 15°C/min to 280°C, and then held for 15 min.

RESULTS AND DISCUSSION

GC-MS analysis of the defensive fluid of *T. slevini* indicated the secretion to be a mixture of low molecular weight carboxylic acids and hydrocarbons (Figure 1, Table 1), a combination frequently encountered in carabid beetles.

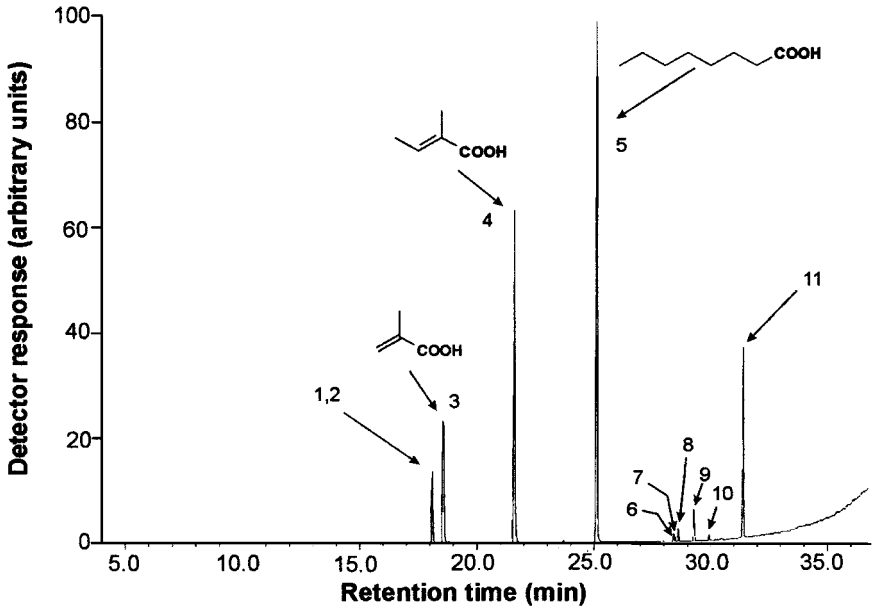


FIG. 1. Reconstructed gas chromatogram obtained from GC-MS analysis of a dichloromethane extract of the defensive secretion of *Trachypachus slevini* (see Table 1 for peak identifications). A fused-silica column (30 m × 0.25 mm) coated with ZB-FFAP was used. The oven temperature was kept at 40°C for 3 min and raised 6°C/min to 265°C.

Major constituents were identified by comparing their mass spectra and gas chromatographic retention times with those of authentic compounds. The major polar components in the fluid were identified as methacrylic and tiglic acids. However, the most abundant constituent was octanoic acid (caprylic acid). Although this is a carboxylic acid, the long alkyl chain renders it relatively nonpolar. Octanoic acid is not common in arthropods, but is known as a secondary or minor component of the secretions of the whip scorpion, *Mastigoproctus giganteus* (Lucas) (Eisner et al., 1961) and some darkling beetles (Tenebrionidae) (Tschinkel, 1975), which use a similar system for defense. In the case of *M. giganteus*, individuals spray a stream of acetic acid containing 5% octanoic acid. Many arthropods combine a polar irritant with nonpolar compounds to afford a more potent defensive mixture. The most likely role of nonpolar compounds is the transport of polar toxins, allowing penetration of the waxy epicuticle of arthropod predators (Eisner et al., 1961). It is likely that octanoic acid functions the same way in *T. slevini*, in relation to the polar components methacrylic and tiglic acids, as it does in *M. giganteus* for acetic acid.

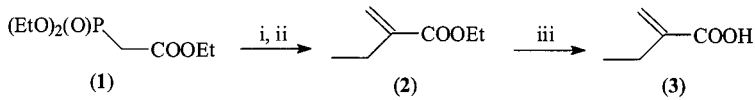
TABLE 1. VOLATILE COMPOUNDS CHARACTERIZED FROM THE PYGIDIAL GLAND CONTENTS OF *Trachypachus slevini*

Peak No.[a]	Compound	Relative amount[b]
1	isovaleric acid	12
2	2-methylbutyric acid	2
3	methacrylic acid	28
4	tiglic acid	68
5	octanoic acid (caprylic acid)	100
6	nonanoic acid	2
7	(*Z*)-9-tricosene[c]	1
8	(*Z*)-7-tricosene[c]	3
9	unidentified	7
10	unidentified	1
11	(*Z*)-9-pentacosene[c]	37

[a] Peak numbers refer to chromatographic peaks in Figure 1.
[b] There was no qualitative variation, however, some quantitative variation in the composition was observed. The values listed here pertain to one sample. The given amounts of components are relative to octanoic acid, which is listed as 100.
[c] Gas chromatographic retention times and corresponding infrared spectra indicate the configuration of the double bond to be *cis*.

In addition, the pygidial gland contents of *T. slevini* show several unsaturated hydrocarbons such as (*Z*)-9-pentacosene. Such long-chain hydrocarbons have been reported from secretions of Hymenoptera (Calam, 1969) but not from beetles. The longest unsaturated hydrocarbon previously reported from beetles is 1-nonadecene (Tschinkel, 1975).

The pygidial gland chemistry of *T. gibbsii* is unique in having the combination of methacrylic and ethacrylic acids as its two major components (Figure 2, Table 2). The later has been reported only rarely from arthropod secretions (Waterhouse and Wallbank, 1967; Moore and Wallbank, 1968; Benn et al., 1973). Identification of ethacrylic acid solely on the basis of its EI mass spectrum is not straightforward since many monounsaturated acids of molecular formula $C_5H_8O_2$ that have been characterized from arthropod secretions produce similar spectra. In order to confirm its identification, and those of a few other compounds that appeared to be esters of ethacrylic acid, we undertook the synthesis of ethacrylic acid according to the reaction steps illustrated in Scheme 1.



(i) NaH, EtI, THF, room temperature; (ii) NaH, $(CH_2O)_n$, Et_2O, 0–20°C, 1.5 hr; (iii) NaOH, H_2O, MeOH, 100°C, 4.5 hr.

SCHEME 1.

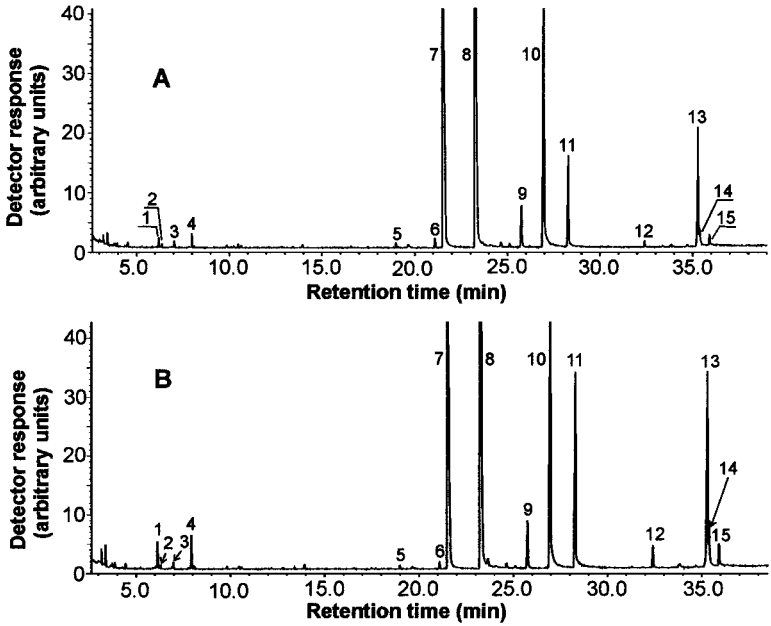


FIG. 2. A. Reconstructed gas chromatogram obtained from a glandular extract of *Trachypachus gibbsii female* by GC-MS analysis. B. Reconstructed gas chromatogram obtained from a glandular extract of *Trachypachus gibbsii male* by GC-MS analysis.

Alkylation of triethyl phosphonoacetate (**1**) with ethyl iodide in the presence of a base, followed by the Horner-Wadsworth-Emmons reaction with paraformaldehyde, gave ethyl 2-ethylacrylate (**2**) (Wadsworth and Emmons, 1961; Jew et al., 1997). Subsequent hydrolysis of **2** gave ethacrylic acid (**3**). The EI mass spectrum and GC retention time of the synthetic product on an FFAP column were indistinguishable from those recorded from the natural product. Moreover, this identification was confirmed by the GC-IR studies (Figure 3).

Similarly, the isopropyl and phenylethyl esters of methacrylic and ethacrylic acids were synthesized and their properties were compared with those recorded for the natural materials. To the best of our knowledge, isopropyl methacrylate, isopropyl ethacrylate, phenethyl methacrylate, and phenethyl ethacrylate have not been previously identified as secondary metabolites from any source.

Several unsaturated hydrocarbons were found in the pygidial gland fluids of *T. slevini* and *T. gibbsii*. Iodine-catalyzed addition of dimethyl disulfide (DMDS) followed by GC-MS analysis of the adducts was carried out (Francis and Veland, 1981). GC-MS analysis of glandular extracts of *T. gibbsii* showed the presence of two pentacosenes and a tricosene. The two adducts derived from pentacosenes showed their molecular ion at m/z 444. The first-eluting isomer showed two peaks

TABLE 2. VOLATILE COMPOUNDS CHARACTERIZED FROM THE PYGIDIAL GLAND CONTENTS OF *Trachypachus gibbsii*

Peak No.[a]	Compound	Relative amount[b]	
		Female	Male
1	isopropyl methacrylate	1	3
2	3-hexanone	trace	trace
3	2-hexanone	1	1
4	isopropyl ethacrylate	1	3
5	isobutyric acid	1	trace
6	2-methylbutyric acid	1	1
7	methacrylic acid	85	73
8	ethacrylic acid (**3**)	100	100
9	2-phenylethanol	7	7
10	2-phenylethyl methacrylate	49	50
11	2-phenylethyl ethacrylate	14	33
12	(*Z*)-7-tricosene[c]	1	3
13	(*Z*)-9- pentacosene[c]	16	31
14	(*Z*)-7-pentacosene[c]	2	7
15	a pentacosadiene[d]	1	3

[a] Peak numbers refer to those given in Figure 2a and 2b.
[b] One sample per sex analyzed.
[c] Gas chromatographic retention times and corresponding infrared spectra indicate the configuration of the double bond is *cis*.
[d] Position and configuration of the double bonds undetermined.

at m/z 271 and 173, while the later-elution isomer showed signals at m/z 299 and 145 indicating the compounds to be 9- and 7-pentacosene, respectively. According to the intensities, the 9-pentacosene is the more abundant isomer. Similarly, the adduct derived from tricosene gave the molecular ion at m/z 416 and fragments at m/z 271 and 145 indicating a double bond at position 7. The two tricosenes from glandular extracts of *T. slevini* were found to be 7- and 9-tricosene, with 7-tricosene the more abundant isomer. The pentacosene was characterized as 9-pentacosene.

Both *T. slevini* and *T. gibbsii* have major compounds that are identical, or biosynthetically similar to, those found in the majority of carabids, and can be grouped within the same chemical classes used by Moore (1979) for Carabidae. *Trachypachus* shares only octanoic and nonanoic acids with some Hydradephaga. These compounds are rarely found in aquatic beetles, and such occurrences are presumed to be derived within dytiscids [e.g., Colymbetinae: *Platambus maculatus* (L.), *Ilybius ater* (Degeer); Laccophilinae: *Laccophilus minutus* (L.)] (Dettner, 1985). While this might provide some evidence that trachypachids fall within Dytiscidae, it does not corroborate morphological evidence that trachypachids are related to hydradephagans, as that evidence clearly suggests trachypachids

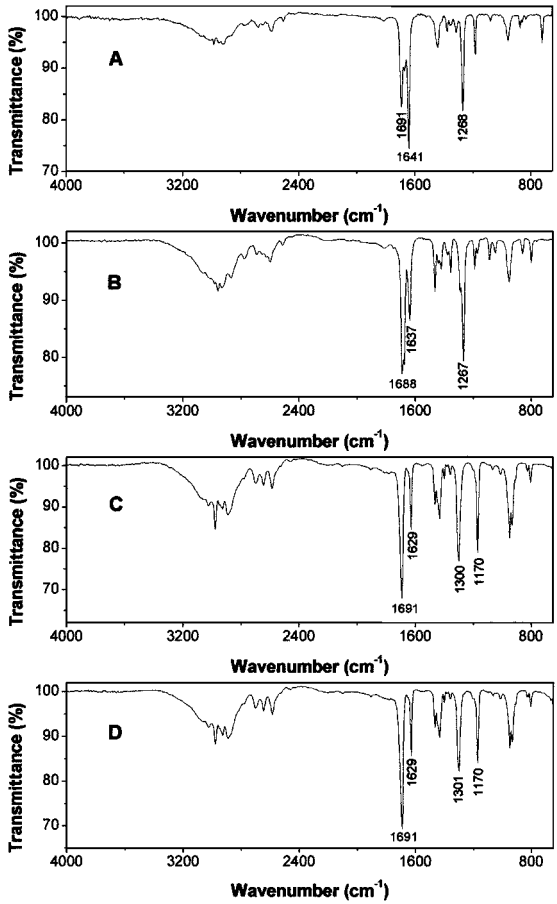


FIG. 3. Condense-phase FTIR spectra recorded by GC-FTIR spectrometry of authentic senecioic acid (A), angelic acid (B), and ethacrylic acid (C), and that corresponding to peak No. 8 (Figure 2) of the chromatogram recorded from the *Trachypachus gibbsii* defensive secretion (D). Resolution = 8 cm^{-1}.

are outside of the family Dytiscidae (Bell, 1966; Hammond, 1979; Roughley, 1981).

A third species, *Trachypachus holmbergi* Mannerheim, is still under study, but preliminary results indicate that the well-known terpene aldehyde citral, and an unidentified sulfur compound are present. Both of these are distinct from compounds found in the two species discussed here. Sulfur compounds are known from a scattering of adephagan taxa [e.g., *Amphizoa* (Dettner, 1990) and various carabids (unpublished data)]. Citral is not known in adephagan beetles but is

reported from Staphylinidae (Blum, 1981). Citral is biosynthetically distant from the aromatic aldehydes (e.g., *p*-hydroxybenzaldehyde) that are commonly found in hydradephagan species.

On the basis of our analyses, each of the three *Trachypachus* species is distinctive in its defensive chemical composition. However, *T. slevini* and *T. gibbsii* both use unsaturated and saturated carboxylic acids in conjunction with hydrocarbons. Ecologically, they all appear to be similar. All three North American species are decidedly terrestrial, found burrowing through loose soil and pine duff (Shull et al., 2001). Given that there is no obvious ecological differentiation in the genus and that the fossil record indicates that this is an ancient group, two scenarios can account for the chemical differentiation. Either the rate of change in the chemical constituents for *Trachypachus* species is relatively high or the divergence of their most recent common ancestor is ancient.

There is no evidence that the rate of character change in general for *Trachypachus* species is elevated as compared to ecologically similar species of Carabidae. The four *Trachypachus* species are morphologically distinct (body form and genitalia) but not exceptionally different. The relative morphological difference is typical, or even somewhat less, from what is commonly recognized between species within carabid genera such as *Bembidion* or *Pterostichus*.

The age of divergence between *Trachypachus* species can be placed in relative terms and broadly estimated using defensive chemical data. In carabid groups with reasonably densely sampled species-level defensive chemical data, those thought to be relatively recent radiations (e.g., *Pterostichus*, Harpalini, Platynini) are remarkably uniform in composition (Dazzini-Valcurone and Pavan, 1980; Will et al., 2000). Older, relictual groups (Erwin, 1985) [e.g., Carabini (Dazzini-Valcurone and Pavan, 1980)] and Gondwanian age groups [e.g., Loxandrini, Pterostichini auctorum (Will, 2000; Will et al., 2000)] generally show clear chemical differentiation. Chemical differences in extant species of *Trachypachus* are consistent with a divergence from their common ancestor during the Cretaceous, somewhere between 125 mya (separation of Australia, India, and Antarctica from Africa and South America) and 50 mya in the Eocene (proposed timing of transberingial dispersal of *Trachypachus*) (Erwin, 1985).

Even though each of the species is distinct chemically, the classes of compounds in *Trachypachus* are more similar to those known from Carabidae than from Hydradephaga. As it is not yet clear whether these similarities are apomorphic or plesiomorphic, we cannot resolve the relationship of trachypachids with other adephagan families on the basis of this evidence. However, on chemical grounds alone, it does allow the possibility that *Trachypachus* is more closely related to Carabidae than to any hydradephagan family. As suggested by Moore (1979), the defensive chemical evidence must be combined with the growing body of data for Adephaga to determine the most likely set of relationships for the included taxa.

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