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Research

Chemosystematics Using Cuticular Compounds: A Powerful Tool to Separate Species in Mediterranean Dung Beetles (Coleoptera: Geotrupidae)

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Abstract

The use of chemical characters to infer a phylogeny is known to be promising to ascertain phylogenetic relationships in controversial groups. Dung beetle classifications containing the Geotrupidae family, based on morphological characters and genes, are debated with respect to the subfamilies, such as the Bolboceratids. In our study, we used different approaches to generate and compare the Geotrupidae phylogenies based on genetics and chemotaxonomy. Cuticular compounds were analyzed for 12 species of Mediterranean dung beetles to build a chemical phylogeny. In addition, mitochondrial and nuclear marker concatenation have been used to elaborate the molecular phylogeny. Using the cuticular compound continuous data, our results showed that each species was associated with a specific chemical pattern and that all individuals belonging to the same species displayed a similar chemical blend. The most distant species was *Bolbelasmus gallicus*, with an evident distinction from the other species due to several compounds. The maximum parsimony tree showed that all genera belonging to a Geotrupidae subfamily were grouped in the same clade, with *B. gallicus* species isolated in another clade, similar to the chemotaxonomy grouping. A strong positive correlation between chemotaxonomy and genetic phylogeny has been demonstrated, underlying a genetic basis for cuticular hydrocarbon variations. Our results are congruent with previous studies using morphological or genetic data. Our results also showed that only 10 compounds can be used to distinguish at least six species of dung beetle and that chemotaxonomy could become a useful and affordable tool to determine phylogenetic relationships in insects.

Key words: Geotrupidae, Bolboceratidae, chemotaxonomy, phylogeny

The cuticle of insects, including the Geotrupidae dung beetles (Coleoptera), is rich in various chemical compounds, primarily hydrocarbons. Several studies have reported the role of cuticular hydrocarbons in the intraspecific communication and mating behavior in Coleoptera (Peterson et al. 2007, Geiselhardt et al. 2009, Ming and Lewis 2010), including the recognition of sexual partners in dung beetles (Ortiz-Domínguez et al. 2006). Hydrocarbons are also used by phoretic mite species as chemical cues for host recognition (Krantz et al. 1991; Krantz and Royce 1994; Takaku et al. 1994; Niogret et al. 2006, 2010). Golden et al. (1992) and Nikolova et al. (1999) noted that the hydrocarbon composition of Coleopteran cuticle was not affected by their diet, excluding effects of environmental factors on the cuticle hydrocarbon variability.

Chemotaxonomy is the use of chemical characters, such as cuticular compounds (CCs), to infer phylogeny. These CCs were used to discriminate between different species in many social and solitary insects (Kaib et al. 1991; Chapman et al. 1995; Blum et al. 2000; Haverty et al. 2000, 2005; Page et al. 2002; Dronnet et al. 2006; Dapporto 2007; Baracchi et al. 2009). Jacob and Hanssen (1986) published one of the first studies demonstrating the use of CCs as taxonomic characters for the chemotaxonomy in Coleoptera. Since then, various studies on Coleoptera families showed that these cuticular characters were species specific within a family, such as in Tenebrionidae (Lockey 1992), Curculionidae (Page et al. 1997, Howard and Perez-Lachaud 2002), Staphylinidae (Lusebrink et al. 2007), and Scarabaeidae (Fletcher et al. 2008).

According to the World Scarabaeidae database, the Geotrupidae family Latreille, 1802 includes 27 genera and more than 360 species distributed in temperate, subtropical, and Asian tropical regions (Schoolmeesters 2010). Most Coleopteran classifications, including

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those for the Geotrupidae family, are based on adult and/or larval morphological characters, but there is still debate regarding the position of various subfamilies. According to some authors, the Geotrupidae family consists of three subfamilies: Geotrupinae, Lethrinae, and Taurocerastinae (Scholtz and Browne 1996, Browne and Scholtz 1999). However, the situation of the Bolboceratidae family is controversial, alternatively considered as a subfamily of the Geotrupidae (Lawrence and Newton 1995, Smith 2006) or as a separate clade (Browne and Scholtz 1999, Verdú et al. 2004). Since the 1950s, dung beetles belonging to the Geotrupidae family were more intensively studied, but only a few papers focused on phylogenetic classifications based on molecular tools (Carisio et al. 2004, Smith 2006, Cunha et al. 2011). To our knowledge, only one of these studies has considered the Bolboceratidae family in their sampling, and they supported its placement in the Geotrupidae family (Smith 2006).

In the present article, we studied 12 species of dung beetle from the Western Mediterranean Basin, including a Bolboceratid species, using cuticular chemical characters as a classification tool. We also compared this chemotaxonomy with a phylogeny based on more conventional genetic markers. To our knowledge, this is the first study to combine both chemotaxonomy and genetics to generate and to compare Coleoptera phylogenies.

Materials and Methods

Sampling

Insects were collected from multiple sites within the Mediterranean region using pitfall traps (CSR model according to Lobo et al. 1988 and Veiga et al. 1989) baited with fresh cattle dung (see Table 1 for locations). Twelve species of dung beetles were analyzed. Eleven species belonging to the Geotrupidae family were morphologically identified (Baraud 1992) as the following species: *Anoplotrupes stercorosus* (Scriba, 1791), *Geotrupes mutator* (Marsham, 1802), *Geotrupes spiniger* (Marsham, 1802), *Geotrupes stercorarius* (Linnaeus, 1758), *Jekelius nitidus* (Jekel, 1865), *Sericotrupes niger* (Marsham, 1802), *Thorectes baraudi* López-Colón, 1981, *Thorectes lusitanicus* (Jekel, 1865), *Trypocopris pyrenaeus* (Charpentier, 1825), *Trypocopris vernalis* (Linnaeus, 1758), and *Typhaeus typhoeus* (Linnaeus, 1758). Unfortunately, only *Bolbelasmus gallicus* (Mulsant, 1842) belonging to the Bolboceratidae family have been collected in our sampling area.

Chemical Analysis

Live beetles were cleaned with distilled water to remove potential dirt and then dried. To avoid cuticular modification induced by stress from trapping and cleaning protocols, the beetles were placed in small plastic containers and left undisturbed for 4 h after manipulation. Beetles were then frozen for 1 h at –25°C, and their abdomens were individually soaked in 2-ml pentane for 5 min. Gas chromatography linked to a mass spectrometer (GC–MS) was used to identify CCs extracted by the solvent. The GC–MS (Shimadzu QP2010 Plus MS coupled with a Shimadzu GC-2010 GC; Shimadzu, Kyoto, Japan) was set with the following parameters: electron impact: 70 eV, 40–330 amu, injector split-splitless at 270°C, oven temperature ramped from 100 to 300°C at 5°C/min and equipped with an SLB-TM-5-MS (Supelco) column, 30 m × 0.25 mm × 0.25 µm film thickness. CCs were identified by comparing mass spectrometers to NIST05 library, chemical standards, and retention index from 'The Pherobase' (El-Sayed 2012). The number of replicates varied per species: 7 *A. stercorosus*, 11 *B. gallicus*, 6 *G. mutator*, 4 *G. spiniger*, 12 *G. stercorarius*, 8 *J. nitidus*, 5 *S. niger*, 9 *Th. baraudi*, 11 *Th. lusitanicus*, 12 *Tr. pyrenaeus*, 4 *Tr. vernalis*, and 6 *Ty. typhoeus*. Compounds representing more than 0.1% of the chemical profile for at least one species were selected and ranked according to their retention index. The relative abundance of each compound was calculated by integrating the area of each peak.

Chemotaxonomy

To allow analysis of individual chemical profiles, the relative areas of peaks were converted to percentage of the total amount of the selected compounds. To visualize pattern differences among species and the 95% confidence ellipses around species, the relative areas of the 69 selected compounds were subjected to a non-metric multidimensional scaling (NMDS), and a cluster analysis using the average linkage (between individuals) method and Euclidean distance among chemicals as a dissimilarity measure using R 2.10.1 software (R Development Core Team 2010), with vegan function and Bray–Curtis index. The similarity percentage analysis (SIMPER) and the analysis of similarities (ANOSIM) were performed on Past 1.99 (Hammer et al. 2001).

DNA Extraction and PCR Amplifications

Total genomic DNA was extracted from one leg using DNeasy Blood and Tissue Kit (QIAGEN, Hombrechtikon, Switzerland) with slight modifications. After solvent extraction of the CCs, beetles were stored in dry conditions. With the intention of improving DNA extraction quality from dry tissues, and to enable more efficient lysis, we modified the conditions of the incubation step with proteinase K. The extracted tissues therefore were incubated for

Table 1. Locations and elevation of the pitfall traps used to catch the dung beetles in the Mediterranean region

Country and region	Location name	Location coordinates	Elevation	Collected species
France and Ariège	Aulus-les-Bains (col d'Agnes)	42°47′N 01°22′E	1,570 m	*Geotrupes stercorarius*
France and Gard	Valleraugue (mont Aigoual)	44°07′N 03°35′E	1,500 m	*Trypocopris pyrenaeus*
	Anduze	44°03′N 03°59′E	250 m	*Typhaeus typhoeus*
	Junas	43°46′N 04°07′E	60 m	*Bolbelasmus gallicus*
	Souvignargues	43°48′N 04°07′E	100 m	*B. gallicus*
France and Hérault	Le Caylar	43°51′N 03°19′E	745 m	*Anoplotrupes stercorosus*, *Geotrupes mutator*, *Trypocopris vernalis*
France and Lozère	Florac	44°19′N 03°35′E	600 m	*Sericotrupes niger*
France and	Font Romeu	42°30′N 02°03′E	1,600 m	*A. stercorosus*, *G. stercorarius*, *Teuchestes fossor*
Pyrénées-Orientales	Mosset (col de Jau)	42°41′N 02°15′E	1,515 m	*A. stercorosus*, *G. stercorarius*, *Tr. pyrenaeus*
	Le Perthus	42°28′N 02°51′E	300 m	*G. mutator*, *Geotrupes spiniger*, *S. niger*
Spain	Algesiras	36°09′N 5°35′W	240 m	*Thorectes lusitanicus*
	Cabañeros National Park	39°24′N 0°35′W	800 m	*Jekelius nitidus*, *Thorectes baraudi*

10 min at 72°C with 20 µl of proteinase K and then kept overnight at 55°C.

Mitochondrial DNA

A 469-base pairs (bp) fragment of the gene encoding Cytochrome Oxidase I (COI) was amplified using primers C1-J-2441 and TL2-N-3014 described in Simon et al. (1994). Polymerase chain reactions (PCR) were performed using the following conditions: initial denaturation for 5 min at 95°C; 40 cycles of 94°C for 1 min, 45–50°C for 1 min, and 72°C for 40 s; and 10-min final extension at 72°C.

Nuclear DNA

An 822-bp fragment of the nuclear gene encoding 18 S Ribosomal marker was amplified with primers 5'18 S and 18 l described in Maddison et al. (1999). We obtained partial amplifications, so we used 500-bp fragment for the beginning of the gene (5'18 S primer) and 322 bp for the end of the gene (18 l primer) to build an 822-bp 18 S nuclear gene fragment. PCR was performed using the following conditions: initial denaturation for 5 min at 95°C; 35 cycles of 94°C for 30 s, 54–56°C for 30 s, and 72°C for 2 min 10 s, and 10-min final extension at 72°C.

Both amplifications (mitochondrial and nuclear fragments) were carried out on a thermocycler (Biometra, Archamps, France) and performed in a volume of 25 µl: 2.5 µl of 10× buffer, 2.5 µl $MgCl_2$ (25 mM), 1 µl primers (10 µM), 0.3 µl dNTPs (20 nM), 1 µl DNA, and 1U of Promega *Taq* polymerase.

Sequencing and Phylogenetic Trees

PCR products were cleaned and sequenced either by a commercial facility (www.lgcgenomics.com) or by using exosapit purification: a blend of 15 µl PCR DNA and 2 µl exosapit solution was put in a Biometra thermocycler (15 min at 37°C following by 15 min at 80°C). All sequencing was performed in both directions. Sequence alignments were done with the BioEdit 7.0.5 software (Raleigh, NC; Hall 1999) by comparing with a previous sequence of COI gene fragment from *G. stercorarius* (AY039377—Villalba et al. 2002) for the mitochondrial gene and a previous sequence of 18 S gene fragment from *G. spiniger* (AY745561.1—Caterino et al. 2005) for the nuclear gene.

Phylogenetic analyses were carried out using an alignment of the concatenation of both sequenced fragments. All positions containing gaps were treated as missing data. Pairwise distances between haplotypes were obtained under the assumptions of the maximum composite likelihood model, and the distance-based tree was inferred using the neighbor-joining (NJ) method (MEGA 4.1 software; Tamura et al. 2007). Tree robustness was tested under bootstrap analysis (1,000 replicates). Numbers of replicates per sequenced species (n = 41 individuals) were the following: 5 *A. stercorosus*, 4 *B. gallicus*, 3 *G. mutator*, 3 *G. spiniger*, 4 *G. stercorarius*, 2 *J. nitidus*, 3 *S. niger*, 3 *Th. baraudi*, 3 *Th. lusitanicus*, 5 *Tr. pyrenaeus*, 1 *Tr. vernalis*, 3 *Ty. typhoeus*. *Teuchestes fossor* (Linnaeus, 1758) (Coleoptera: Aphodiidae) was selected as an outgroup to root our trees due to its relative distance to the Geotrupinae and the Bolboceratid species (our results have confirmed that those two groups were two distinct families).

Results

Chemical Analysis

In our analysis, we selected 69 CCs with substantial quantitative or qualitative differences providing enough information to discriminate between the species. Representative chromatograms for each species are shown in Fig. 1, and the identification and proportion of the 69 CCs are described in Table 2. We did not find any qualitative or quantitative chemical differences between males and females in any of the species studied. The ANOSIM allowed us to distinguish the species in 95.5% of the cases ($P < 0.005$; included 41% with $P < 0.0001$; data not shown), i.e., there is a significant separation between all the species but four, between *A. stercorosus* and *G. stercorarius*, between *Tr. vernalis* and *G. mutator*, and between *Tr. vernalis* and *G. stercorarius*. The estimated contribution of single compounds using a SIMPER analysis by pooling all species to the observed variation between species showed that the 10 first compounds responsible for 44% of the observed total variance were in the following order (Fig. 1): CC25 (pentacosene), CC54 (squalene), CC24 (5,9-pentacosadiene), CC36 (methylhexacosane), CC39 (ethylpentacosane), CC3 (heneicosene), CC40 (heptacosene), CC4 (heneicosane), CC44 (heptacosane), and CC27 (pentacosane; data not shown). CC25, CC24, CC39, CC44, and CC27 were present in all species, but at different ratios. CC25 was one of the major peaks in *A. stercorosus*, *G. mutator*, *G. stercorarius*, and *Tr. vernalis*; CC24 was the highest in *Th. lusitanicus* and predominant in *J. nitidus*; CC44 in both *Trypocopris* species; and CC27 was predominant in *Tr. pyrenaeus*. Three compounds, CC54, CC36, and CC40, were present in all species with the exception of *B. gallicus*. Moreover, CC54 was the major peak in *S. niger* and *G. spiniger*; CC36 and CC40 were the highest in *Ty. typhoeus* and *Tr. pyrenaeus*, respectively. CC3, the major peak in *B. gallicus*, was present only in three other species (*J. nitidus*, *Tr. pyrenaeus*, and *Th. lusitanicus*) and in a very low ratio. CC4, the second major peak in *B. gallicus*, was absent or present at a very low level in all the remaining species (see Table 2).

NMDS analysis (Fig. 2) on quantitative variations of CCs revealed that the individuals were regrouped by species (Fig. 2a), and all species were clustered by genus (Fig. 2b) with low individual variability (stress = 0.14). Each species was associated with a specific chemical pattern, even within the same genus. For instance, within the genus *Trypocopris*, several differences could be described between the two studied species. The major compound of *Tr. vernalis*, CC25 (14.5%), represented only 1% of the chemical profile of *Tr. pyrenaeus*, whereas the major compound of *Tr. pyrenaeus*, CC40 (10.8%), was only present at 4.3% in *Tr. vernalis*. Moreover, qualitative differences were also species characteristic. Several compounds, e.g., CC3, CC4, and CC7, were absent in *Tr. vernalis*, but present in *Tr. pyrenaeus*.

According to NMDS analysis, the most distant species was *B. gallicus* with an evident distinction from other species. Indeed, the lighter compounds, CC3 and CC4, were responsible for an average of 21.1% of the divergence between *B. gallicus* and the other species (SIMPER analysis, data not shown). *Typhaeus typhoeus* and *J. nitidus* were also among the most divergent species in NMDS results. The two *Thorectes* species and *S. niger* were easily distinguishable from the others. The two *Trypocopris* species were very close. *Geotrupes stercorarius* and *G. mutator* were undistinguishable species in the *Geotrupes* genus, whereas *G. spiniger* (considered to be a close relative of *G. stercorarius*) was well distinct.

The cluster analysis (Fig. 3) provided a dendrogram, in which relative distances among the 12 studied species were determined by the average distances among their chemical profiles. We observed the same topology using the NMDS analysis. *Bolbelasmus gallicus* was very distant from all the other species. As in the NMDS analysis, several species can be dissociated from each other, with all their specific individuals in the same cluster: *Ty. typhoeus*, *J. nitidus*, *Th. lusitanicus*, and *Th. baraudi*. Also, individuals of *Tr. pyrenaeus* were recovered as a distinct and separate clade, whereas individuals of *Tr. vernalis* were distantly scattered across the topology.

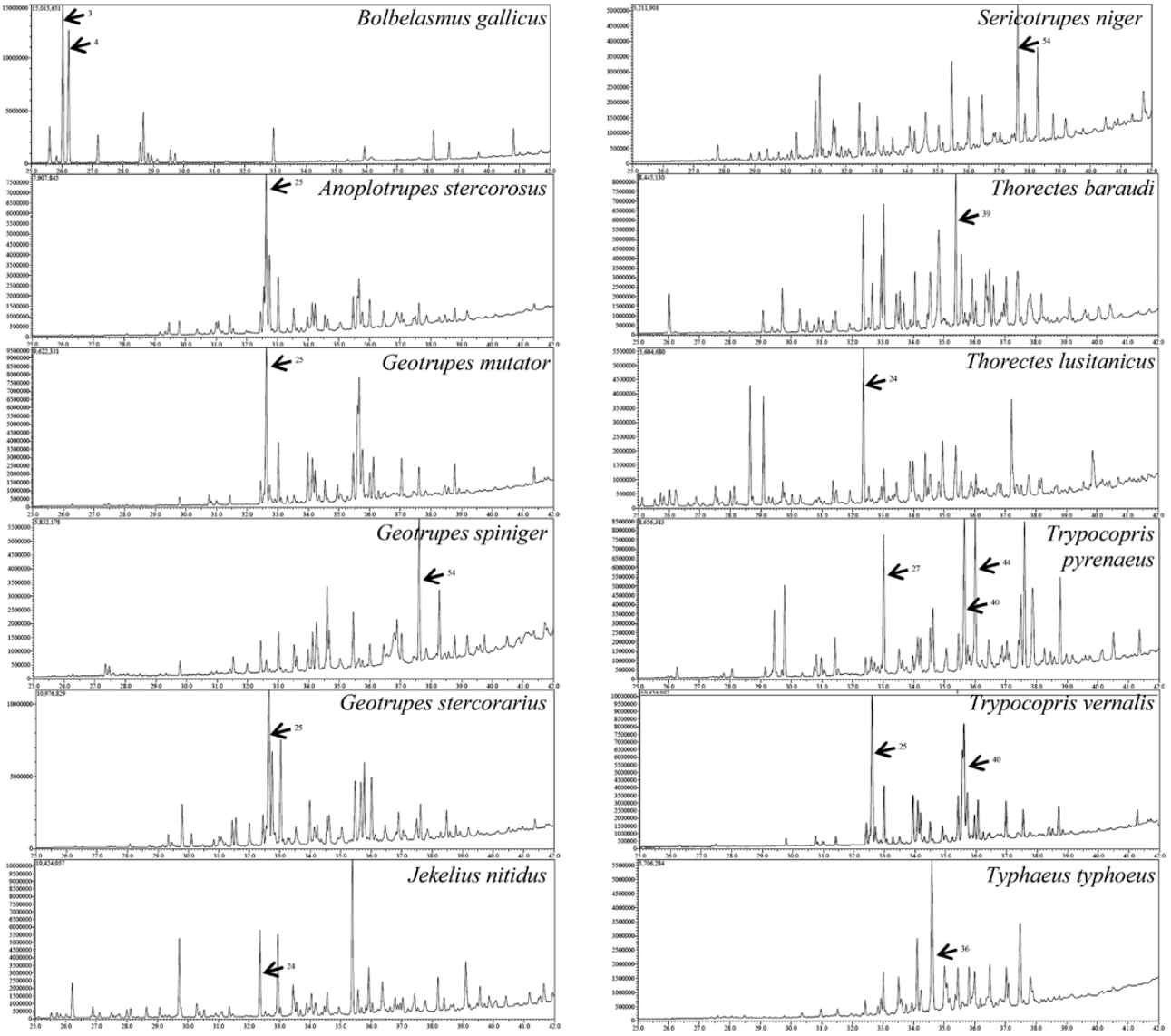

Fig. 1. Representative gas chromatography spectra of cuticular compounds from the 12 studied species of dung beetles.

Moreover, using only the 10 compounds responsible for 44% of the observed total variance described earlier, we could still distinguish 6 of the 12 using either NMDS or cluster analysis: *B. gallicus*, *Ty. typhoeus*, *J. nitidus*, *Tr. pyrenaeus*, *Th. lusitanicus*, and *Th. baraudi* (data not shown).

Sequence Analysis

The sequence alignment was done on the concatenation of gene fragments encoding for both mitochondrial COI and nuclear 18S, with a total 1,291 bp. Interestingly, sequences from individuals *B. gallicus* were distinguishable by the presence of indels (18 bp) with respect to all the remaining analyzed species. The mean base composition of the concatenation of both genes was as follows: T = 28.8%; C = 22.2%; A = 26.7%; G = 22.3%. For the mitochondrial marker, sequence divergence within species ranged from 0 to 4.7% (data not shown). Inside the Geotrupidae family, excluding *B. gallicus*, the divergence among species averaged 14.5%. Moreover, between Geotrupidae and Bolboceratidae, the latter represented by *B. gallicus*, this divergence was high (32.3% on average), which was even higher than sequence divergence with the outgroup (*Te. fossor*; Aphodiidae; 27.1%). Sequence divergences were weaker for the nuclear marker (data not shown) than for the mitochondrial marker: they ranged from 0 to 1.6% within species, averaged at 0.3% inside Geotrupidae family, and were 2.7 and 2.1% between Geotrupidae and Bolboceratidae and between Geotrupidae and outgroup, respectively.

Phylogenetic Tree

Using the concatenation of the mitochondrial CO1 and the nuclear 18S genes, and a maximum parsimony analysis, the consistency index and the retention index were 0.55 and 0.86, respectively. There were a total of 1,270 positions in the final data set, of which 212 resulted to be informative. The phylogenetic tree inferred using the NJ and maximum parsimony methods yielded the same topology (NJ tree on Fig. 4).

The monophyly of all analyzed genera was tested by bootstrap values (BV) varying from 15 to 100% (Fig. 4). All species, but two

Table 2. Cuticular compound composition of the 12 studied species of dung beetles

	RI	Name	*Anoplotrupes stercorosus*	*Bolbelasmus gallicus*	*Geotrupes mutator*	*Geotrupes spiniger*	*Geotrupes stercorarius*	*Jekelius nitidus*
CC1	2069	Heneicosanol	0.00 ± 0.00	4.78 ± 6.28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.19
CC2	2081	Heneicosene	0.23 ± 0.51	1.04 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC3	**2092**	**Heneicosene**	**0.00 ± 0.00**	**21.29 ± 4.60**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.01 ± 0.03**
CC4	**2103**	**Heneicosane**	**0.31 ± 0.35**	**16.68 ± 3.98**	**0.19 ± 0.24**	**0.00 ± 0.00**	**0.48 ± 0.44**	**0.40 ± 1.14**
CC5	2118	(Z)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.00 ± 0.00	1.09 ± 0.87	0.00 ± 0.00	0.00 ± 0.00	0.37 ± 0.95	0.00 ± 0.00
CC6	2155	Ethyl octadecenoate	0.00 ± 0.00	8.77 ± 5.16	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.54	0.02 ± 0.07
CC7	2200	Docosane	0.03 ± 0.08	0.32 ± 0.10	0.26 ± 0.41	0.19 ± 0.27	0.20 ± 0.31	0.00 ± 0.00
CC8	2228	Docosene	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC9	2233	Methyldocosane	0.00 ± 0.00	4.27 ± 3.13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.03
CC10	2240	Methyldocosane	0.00 ± 0.00	4.06 ± 2.07	0.07 ± 0.16	0.00 ± 0.00	0.09 ± 0.20	0.12 ± 0.38
CC11	2249	Methyldocosane	0.00 ± 0.00	1.00 ± 0.44	0.09 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC12	2269	Methyldocosane	0.98 ± 0.50	0.63 ± 0.31	0.63 ± 1.53	0.00 ± 0.00	0.99 ± 1.67	1.34 ± 1.00
CC13	2275	9-Tricosene	0.27 ± 0.25	0.10 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.77 ± 0.96	0.03 ± 0.08
CC14	2280	Methyldocosane	0.90 ± 0.59	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.68 ± 1.09	0.02 ± 0.07
CC15	2292	(Z)-5-Tricosene	0.07 ± 0.18	2.77 ± 1.14	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.38	0.43 ± 0.92
CC16	2302	Tricosane	2.55 ± 1.41	1.84 ± 0.59	1.08 ± 0.85	1.14 ± 0.90	4.48 ± 1.56	1.31 ± 2.55
CC17	2320	Trimethyl docosane	0.00 ± 0.00	1.71 ± 1.74	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.07
CC18	2348	Methyl tricosane	0.64 ± 0.48	0.00 ± 0.00	0.07 ± 0.17	0.08 ± 0.17	0.18 ± 0.21	0.34 ± 0.44
CC19	2378	Dimethyltricosane	0.66 ± 0.33	0.19 ± 0.11	0.34 ± 0.48	0.00 ± 0.00	0.72 ± 0.59	0.21 ± 0.15
CC20	2386	Trimethyl docosane	2.40 ± 1.06	0.00 ± 0.00	0.32 ± 0.37	0.08 ± 0.18	1.45 ± 0.69	0.33 ± 0.18
CC21	2393	Trimethyl tricosane	0.22 ± 0.38	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.22	0.31 ± 0.25
CC22	2402	Tetracosane	1.39 ± 1.37	0.37 ± 0.16	0.80 ± 0.48	0.54 ± 0.42	1.67 ± 0.75	1.03 ± 0.28
CC23	2414	Ethyl tricosane	0.00 ± 0.00	0.04 ± 0.03	0.25 ± 0.61	2.77 ± 2.51	1.48 ± 1.62	0.15 ± 0.14
CC24	**2472**	**5,9-Pentacosadiene**	**6.72 ± 1.38**	**0.35 ± 0.12**	**4.66 ± 2.14**	**4.06 ± 1.24**	**4.84 ± 2.02**	**11.10 ± 4.47**
CC25	**2477**	**Pentacosene (Z7 ou Z9)**	**16.02 ± 7.94**	**0.02 ± 0.02**	**17.83 ± 12.89**	**0.71 ± 0.60**	**21.85 ± 12.38**	**0.82 ± 0.55**
CC26	2484	5-Pentacosene	8.15 ± 3.41	0.03 ± 0.03	1.33 ± 1.51	0.20 ± 0.28	5.38 ± 3.79	0.49 ± 0.32
CC27	**2502**	**Pentacosane**	**6.93 ± 3.03**	**6.87 ± 1.81**	**5.72 ± 2.22**	**5.81 ± 1.69**	**9.33 ± 2.43**	**3.71 ± 2.60**
CC28	2507	Methyl-tetracosane	0.61 ± 0.45	0.00 ± 0.00	0.73 ± 0.60	0.70 ± 0.48	0.69 ± 0.58	1.22 ± 0.67
CC29	2535	13-Methylpentacosane	2.27 ± 1.00	0.16 ± 0.08	0.99 ± 0.38	4.30 ± 2.27	1.60 ± 0.63	4.60 ± 1.20
CC30	2540	Methylpentacosane	0.50 ± 0.41	0.02 ± 0.02	0.00 ± 0.00	2.16 ± 0.85	0.26 ± 0.22	2.03 ± 1.33
CC31	2551	5-Methylpentacosane	0.55 ± 0.46	0.00 ± 0.00	0.11 ± 0.28	0.28 ± 0.40	0.09 ± 0.24	1.66 ± 1.05
CC32	2569	5-Methylpentacosane	2.02 ± 0.62	0.11 ± 0.06	3.19 ± 1.42	1.84 ± 0.63	1.78 ± 0.55	2.45 ± 0.96
CC33	2574	3-Methylpentacosane	4.03 ± 1.14	0.06 ± 0.06	0.48 ± 1.18	2.32 ± 0.90	3.06 ± 1.21	3.78 ± 0.95
CC34	2580	Methylpentacosane	2.20 ± 1.45	0.04 ± 0.07	0.59 ± 0.35	3.82 ± 2.57	0.67 ± 0.34	3.04 ± 1.13
CC35	2600	Hexacosane	1.35 ± 0.53	0.33 ± 0.10	2.40 ± 1.01	1.90 ± 1.90	1.42 ± 0.65	4.38 ± 1.30
CC36	**2611**	**Methyl-hexacosane**	**1.75 ± 1.34**	**0.00 ± 0.00**	**0.95 ± 1.26**	**8.15 ± 6.60**	**2.21 ± 1.44**	**5.71 ± 2.03**
CC37	2632	Unidentified hydrocarbon	0.13 ± 0.25	0.03 ± 0.05	0.00 ± 0.00	2.90 ± 1.66	0.08 ± 0.16	0.03 ± 0.11
CC38	2640	Methyl-hexacosane	1.19 ± 1.27	0.17 ± 0.14	2.02 ± 1.83	0.83 ± 0.69	1.40 ± 0.69	1.11 ± 0.68
CC39	**2670**	**Ethylpentacosane**	**6.33 ± 2.65**	**1.02 ± 0.50**	**10.57 ± 3.62**	**5.11 ± 2.94**	**3.47 ± 1.49**	**11.77 ± 2.24**
CC40	**2684**	**Heptacosene**	**3.69 ± 1.26**	**0.00 ± 0.00**	**9.63 ± 6.12**	**4.20 ± 6.31**	**5.17 ± 4.23**	**1.02 ± 0.46**
CC41	2692	Heptacosene	1.04 ± 0.64	0.38 ± 0.60	3.15 ± 1.34	2.33 ± 3.83	2.37 ± 1.33	0.29 ± 0.21
CC42	2694	Dimethylhexacosane	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.10	0.00 ± 0.00	0.17 ± 0.26	2.52 ± 2.08
CC43	2696	Dimethylhexacosane	0.28 ± 0.32	0.00 ± 0.00	0.06 ± 0.15	0.22 ± 0.49	0.11 ± 0.18	3.55 ± 2.39
CC44	**2700**	**Heptacosane**	**4.04 ± 2.58**	**3.06 ± 1.15**	**5.52 ± 1.25**	**2.97 ± 1.08**	**5.27 ± 4.99**	**1.28 ± 2.76**
CC45	2716	Unidentified hydrocarbon	0.07 ± 0.13	0.00 ± 0.00	0.55 ± 0.75	0.16 ± 0.35	0.10 ± 0.21	0.33 ± 0.34
CC46	2739	Methylheptacosane	2.26 ± 1.24	0.05 ± 0.06	1.32 ± 1.15	1.65 ± 0.45	0.99 ± 0.50	2.84 ± 1.31
CC47	2737	Methylheptacosane	0.25 ± 0.27	0.30 ± 0.54	0.94 ± 0.45	0.57 ± 0.75	0.27 ± 0.25	0.87 ± 0.61
CC48	2742	Methylheptacosane	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.24	0.08 ± 0.19	0.00 ± 0.00	1.04 ± 0.60
CC49	2750	Methylheptacosane	0.09 ± 0.15	0.30 ± 0.59	0.71 ± 1.74	0.00 ± 0.00	0.08 ± 0.25	0.00 ± 0.00
CC50	2770	Methylheptacosane	1.02 ± 0.26	0.10 ± 0.12	1.51 ± 1.20	1.94 ± 0.84	0.73 ± 0.54	1.14 ± 0.65
CC51	2780	Methylheptacosane	1.49 ± 0.26	0.07 ± 0.12	3.49 ± 0.66	1.37 ± 1.02	1.21 ± 0.83	7.65 ± 2.37
CC52	2786	Methylheptacosane	0.65 ± 0.52	0.10 ± 0.21	0.00 ± 0.00	0.20 ± 0.19	0.21 ± 0.16	2.87 ± 0.67
CC53	2800	Octacosane	0.28 ± 0.27	0.41 ± 0.15	0.60 ± 0.64	0.34 ± 0.21	0.15 ± 0.23	2.54 ± 1.65
CC54	**2816**	**Squalene**	**7.35 ± 5.42**	**0.00 ± 0.00**	**8.38 ± 8.35**	**17.55 ± 9.80**	**5.61 ± 3.38**	**1.57 ± 1.93**
CC55	2858	Methyloctacosane	1.13 ± 0.96	0.11 ± 0.16	1.13 ± 1.52	0.27 ± 0.59	0.75 ± 0.74	0.65 ± 0.34
CC56	2864	Methyloctacosane	1.09 ± 1.07	3.86 ± 2.18	1.83 ± 2.18	4.73 ± 1.37	1.35 ± 1.34	0.29 ± 0.62
CC57	2893	Nonacosene	0.19 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC58	2900	Nonacosane	1.88 ± 1.10	3.14 ± 0.82	2.68 ± 0.40	2.40 ± 1.69	1.63 ± 1.65	2.69 ± 2.36
CC59	2932	11,13-Dimethyloctacosane C30	0.96 ± 1.09	0.38 ± 0.13	0.72 ± 0.85	2.83 ± 3.13	0.32 ± 0.21	0.93 ± 1.72
CC60	2974	Methylnonacosane	0.33 ± 0.39	0.21 ± 0.15	0.28 ± 0.43	1.24 ± 0.36	0.19 ± 0.48	0.60 ± 0.47
CC61	2981	Methylnonacosane	0.00 ± 0.00	0.72 ± 0.42	0.30 ± 0.41	1.04 ± 0.67	0.05 ± 0.14	0.59 ± 0.35

Table 2. *Continued*

	RI	Name	*Anoplotrupes stercorosus*	*Bolbelasmus gallicus*	*Geotrupes mutator*	*Geotrupes spiniger*	*Geotrupes stercorarius*	*Jekelius nitidus*
CC62	2984	Methylnonacosane	0.00 ± 0.00	0.12 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC63	3000	Triacontane	0.00 ± 0.00	0.22 ± 0.16	0.00 ± 0.00	0.20 ± 0.27	0.01 ± 0.02	0.07 ± 0.21
CC64	3039	Methyltriacontane	0.00 ± 0.00	0.25 ± 0.27	0.07 ± 0.18	0.00 ± 0.00	0.17 ± 0.30	0.00 ± 0.00
CC65	3064	Methyltriacontane	0.00 ± 0.00	0.16 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.29	0.00 ± 0.00
CC66	3079	Methyltriacontane	0.09 ± 0.16	4.25 ± 2.05	0.00 ± 0.00	1.88 ± 2.67	0.16 ± 0.25	0.02 ± 0.06
CC67	3101	Methyltriacontane	0.11 ± 0.19	0.47 ± 0.62	0.22 ± 0.35	1.16 ± 0.77	0.12 ± 0.30	0.13 ± 0.43
CC68	3107	Hentriacontane	0.00 ± 0.00	0.25 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC69	3129	13-Methylhentriacontane	0.30 ± 0.31	0.90 ± 0.88	0.92 ± 0.27	0.79 ± 0.50	0.57 ± 0.60	0.46 ± 0.42
CC1	2069	Heneicosanol	0.00 ± 0.00	0.00 ± 0.00	1.35 ± 1.45	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC2	2081	Heneicosene	0.00 ± 0.00	0.00 ± 0.00	0.58 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC3	**2092**	**Heneicosene**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.07 ± 0.17**	**0.02 ± 0.06**	**0.00 ± 0.00**	**0.00 ± 0.00**
CC4	**2103**	**Heneicosane**	**0.07 ± 0.15**	**0.00 ± 0.00**	**2.47 ± 0.87**	**0.58 ± 0.25**	**0.00 ± 0.00**	**0.00 ± 0.00**
CC5	2118	(Z)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.98 ± 3.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC6	2155	Ethyl octadecenoate	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC7	2200	Docosane	0.13 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.47 ± 0.27	0.00 ± 0.00	0.33 ± 0.38
CC8	2228	Docosene	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.34
CC9	2233	Methyldocosane	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.13	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.20
CC10	2240	Methyldocosane	0.00 ± 0.00	0.00 ± 0.00	3.60 ± 3.17	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.19
CC11	2249	Methyldocosane	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.33
CC12	2269	Methyldocosane	0.33 ± 0.49	1.33 ± 1.17	10.05 ± 2.77	1.00 ± 0.54	0.38 ± 0.44	0.05 ± 0.13
CC13	2275	9-Tricosene	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.35	0.64 ± 0.54	0.38 ± 0.50	0.00 ± 0.00
CC14	2280	Methyl docosane	0.00 ± 0.00	0.12 ± 0.16	0.51 ± 0.52	5.31 ± 2.42	1.29 ± 1.91	0.00 ± 0.00
CC15	2292	(Z)-5-Tricosene	0.27 ± 0.59	0.01 ± 0.04	0.23 ± 0.28	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC16	2302	Tricosane	1.30 ± 0.84	1.79 ± 0.64	3.16 ± 1.68	5.15 ± 1.77	1.91 ± 1.42	0.13 ± 0.33
CC17	2320	Trimethyl docosane	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC18	2348	Methyl tricosane	0.50 ± 0.73	0.75 ± 0.46	1.08 ± 0.73	0.13 ± 0.14	0.00 ± 0.00	0.10 ± 0.24
CC19	2378	Dimethyltricosane	0.13 ± 0.29	0.22 ± 0.14	0.39 ± 0.52	1.39 ± 0.64	0.54 ± 0.62	0.16 ± 0.40
CC20	2386	Trimethyl docosane	0.48 ± 0.69	0.75 ± 0.31	0.40 ± 0.61	1.25 ± 0.53	1.76 ± 2.00	0.26 ± 0.41
CC21	2393	Trimethyl tricosane	0.00 ± 0.00	0.69 ± 0.51	1.46 ± 1.15	0.00 ± 0.00	0.50 ± 0.68	0.00 ± 0.00
CC22	2402	Tetracosane	0.24 ± 0.33	0.70 ± 0.15	2.03 ± 0.58	2.26 ± 0.79	1.81 ± 0.67	0.11 ± 0.17
CC23	2414	Ethyl tricosane	0.13 ± 0.28	1.06 ± 0.56	1.75 ± 0.50	0.36 ± 0.23	0.47 ± 0.54	0.49 ± 0.44
CC24	**2472**	**5,9-Pentacosadiene**	**1.74 ± 2.39**	**8.21 ± 2.56**	**10.70 ± 2.08**	**1.84 ± 0.85**	**5.13 ± 2.49**	**1.26 ± 0.76**
CC25	**2477**	**Pentacosene (Z7 ou Z9)**	**0.79 ± 0.78**	**0.42 ± 0.48**	**0.79 ± 0.52**	**1.03 ± 0.33**	**14.53 ± 8.26**	**0.05 ± 0.13**
CC26	2484	5-Pentacosene	0.25 ± 0.35	1.48 ± 0.61	0.43 ± 0.50	0.92 ± 0.35	1.99 ± 1.50	0.00 ± 0.00
CC27	**2502**	**Pentacosane**	**3.68 ± 1.10**	**5.79 ± 2.21**	**2.81 ± 1.50**	**9.47 ± 1.97**	**7.84 ± 3.62**	**3.43 ± 0.80**
CC28	2507	Methyl-tetracosane	0.00 ± 0.00	0.36 ± 0.23	0.18 ± 0.24	0.00 ± 0.00	0.14 ± 0.28	3.41 ± 1.08
CC29	2535	13-Methylpentacosane	0.66 ± 0.75	2.42 ± 0.98	2.65 ± 0.78	1.77 ± 0.73	0.51 ± 0.60	3.55 ± 1.55
CC30	2540	Methylpentacosane	0.14 ± 0.32	1.75 ± 0.59	0.59 ± 0.36	0.87 ± 0.41	0.46 ± 0.53	2.09 ± 1.06
CC31	2551	5-Methylpentacosane	0.00 ± 0.00	1.67 ± 0.65	0.21 ± 0.32	0.32 ± 0.30	0.08 ± 0.16	0.34 ± 0.39
CC32	2569	5-Methylpentacosane	0.55 ± 0.60	0.59 ± 0.09	2.80 ± 0.60	1.01 ± 0.22	0.39 ± 0.56	0.96 ± 0.86
CC33	2574	3-Methylpentacosane	1.74 ± 0.38	2.97 ± 0.49	1.73 ± 1.07	3.42 ± 0.57	3.93 ± 1.10	5.61 ± 2.98
CC34	2580	Methylpentacosane	0.67 ± 1.14	1.07 ± 0.57	5.23 ± 3.10	0.84 ± 0.36	0.76 ± 0.51	1.61 ± 0.99
CC35	2600	Hexacosane	0.42 ± 0.43	9.70 ± 3.59	3.05 ± 2.56	2.56 ± 0.63	2.30 ± 0.82	0.75 ± 0.42
CC36	**2611**	**Methyl-hexacosane**	**1.97 ± 0.59**	**4.78 ± 1.40**	**2.75 ± 1.57**	**4.13 ± 1.50**	**2.96 ± 1.09**	**20.74 ± 5.89**
CC37	2632	Unidentified hydrocarbon	0.00 ± 0.00	0.03 ± 0.06	6.55 ± 4.73	0.00 ± 0.00	0.35 ± 0.41	0.19 ± 0.47
CC38	2640	Methyl-hexacosane	2.28 ± 0.78	2.65 ± 2.37	3.75 ± 0.72	1.30 ± 0.45	1.55 ± 1.12	7.47 ± 1.98
CC39	**2670**	**Ethylpentacosane**	**3.55 ± 3.05**	**10.71 ± 3.40**	**3.90 ± 2.09**	**3.67 ± 1.50**	**4.29 ± 2.06**	**5.10 ± 2.27**
CC40	**2684**	**Heptacosene**	**2.38 ± 1.35**	**1.56 ± 0.98**	**1.84 ± 0.88**	**10.81 ± 2.07**	**4.25 ± 0.72**	**0.17 ± 0.41**
CC41	2692	Heptacosene	0.67 ± 1.10	0.70 ± 0.24	0.36 ± 0.32	1.43 ± 0.61	1.12 ± 0.77	0.36 ± 0.40
CC42	2694	Dimethylhexacosane	0.21 ± 0.48	0.54 ± 0.14	0.17 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	0.34 ± 0.41
CC43	2696	Dimethylhexacosane	0.00 ± 0.00	2.24 ± 1.74	1.17 ± 0.99	1.61 ± 1.10	1.59 ± 1.59	3.24 ± 1.74
CC44	**2700**	**Heptacosane**	**5.14 ± 1.08**	**1.30 ± 0.91**	**0.89 ± 0.69**	**8.92 ± 2.26**	**7.42 ± 5.00**	**3.21 ± 2.32**
CC45	2716	Unidentified hydrocarbon	0.00 ± 0.00	0.45 ± 0.38	0.61 ± 0.55	0.00 ± 0.00	0.12 ± 0.24	3.35 ± 1.89
CC46	2739	Methylheptacosane	3.00 ± 2.42	3.37 ± 0.69	0.99 ± 0.36	1.75 ± 0.51	0.38 ± 0.76	2.20 ± 2.28
CC47	2737	Methylheptacosane	0.43 ± 0.69	2.22 ± 0.45	0.19 ± 0.20	0.00 ± 0.00	0.24 ± 0.48	3.97 ± 2.16
CC48	2742	Methylheptacosane	0.19 ± 0.42	0.86 ± 0.62	0.11 ± 0.15	0.38 ± 0.18	0.38 ± 0.76	0.14 ± 0.35
CC49	2750	Methylheptacosane	0.08 ± 0.18	0.12 ± 0.13	0.00 ± 0.00	0.17 ± 0.14	0.00 ± 0.00	0.48 ± 0.53
CC50	2770	Methylheptacosane	4.03 ± 1.79	0.29 ± 0.18	1.83 ± 0.54	1.34 ± 0.35	0.81 ± 1.07	0.70 ± 0.78
CC51	2780	Methylheptacosane	5.06 ± 2.95	2.47 ± 0.52	0.79 ± 0.39	2.20 ± 0.46	2.86 ± 1.04	4.78 ± 1.37
CC52	2786	Methylheptacosane	0.37 ± 0.82	2.45 ± 0.45	0.95 ± 0.66	0.47 ± 0.20	0.13 ± 0.25	2.31 ± 0.95
CC53	2800	Octacosane	1.12 ± 0.41	0.48 ± 0.48	0.04 ± 0.13	1.09 ± 0.54	1.06 ± 0.79	0.00 ± 0.00
CC54	**2816**	**Squalene**	**21.25 ± 8.14**	**5.78 ± 0.80**	**4.12 ± 1.27**	**8.81 ± 5.58**	**5.25 ± 1.33**	**10.22 ± 3.19**
CC55	2858	Methyloctacosane	2.32 ± 0.74	5.27 ± 1.67	1.92 ± 0.56	3.99 ± 2.29	1.91 ± 1.30	3.44 ± 0.89

Table 2. *Continued*

	RI	Name	*Anoplotrupes stercorosus*	*Bolbelasmus gallicus*	*Geotrupes mutator*	*Geotrupes spiniger*	*Geotrupes stercorarius*	*Jekelius nitidus*
CC56	2864	Methyloctacosane	10.97 ± 2.25	2.67 ± 2.12	1.56 ± 0.82	0.74 ± 0.34	2.54 ± 1.54	0.66 ± 0.67
CC57	2893	Nonacosene	0.36 ± 0.51	0.00 ± 0.00	0.57 ± 0.67	0.20 ± 0.22	0.14 ± 0.29	0.15 ± 0.24
CC58	2900	Nonacosane	6.32 ± 5.31	0.77 ± 0.55	0.76 ± 0.83	3.67 ± 1.08	5.31 ± 4.74	0.33 ± 0.40
CC59	2932	11,13-Dimethyloctacosane C30	2.77 ± 2.72	1.07 ± 0.70	0.49 ± 0.34	0.37 ± 0.24	0.25 ± 0.50	0.00 ± 0.00
CC60	2974	Methylnonacosane	0.71 ± 0.75	0.00 ± 0.00	0.21 ± 0.30	0.37 ± 0.23	1.66 ± 3.33	0.00 ± 0.00
CC61	2981	Methylnonacosane	1.93 ± 1.49	0.86 ± 0.27	0.00 ± 0.00	0.00 ± 0.00	2.80 ± 5.34	0.00 ± 0.00
CC62	2984	Methylnonacosane	0.25 ± 0.55	0.00 ± 0.00	0.14 ± 0.48	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.11
CC63	3000	Triacontane	0.27 ± 0.37	0.87 ± 0.65	0.14 ± 0.36	0.01 ± 0.05	0.14 ± 0.28	0.00 ± 0.00
CC64	3039	Methyltriacontane	0.17 ± 0.38	0.00 ± 0.00	0.03 ± 0.11	0.00 ± 0.00	1.16 ± 1.91	0.00 ± 0.00
CC65	3064	Methyltriacontane	0.66 ± 0.91	0.00 ± 0.00	0.58 ± 0.94	0.95 ± 0.47	0.20 ± 0.41	0.05 ± 0.11
CC66	3079	Methyltriacontane	3.46 ± 3.60	0.60 ± 0.53	0.94 ± 0.92	0.31 ± 0.35	0.43 ± 0.86	0.37 ± 0.58
CC67	3101	Methyltriacontane	0.94 ± 1.46	0.00 ± 0.00	0.30 ± 0.55	0.00 ± 0.00	0.60 ± 0.69	0.37 ± 0.45
CC68	3107	Hentriacontane	0.00 ± 0.00	0.28 ± 0.64	0.00 ± 0.00	0.04 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
CC69	3129	13-Methylhentriacontane	0.94 ± 0.95	0.73 ± 0.96	0.55 ± 0.43	0.71 ± 0.33	0.99 ± 1.30	0.22 ± 0.40

The compounds responsible for 44% of the observed total variance between the species are in bold.

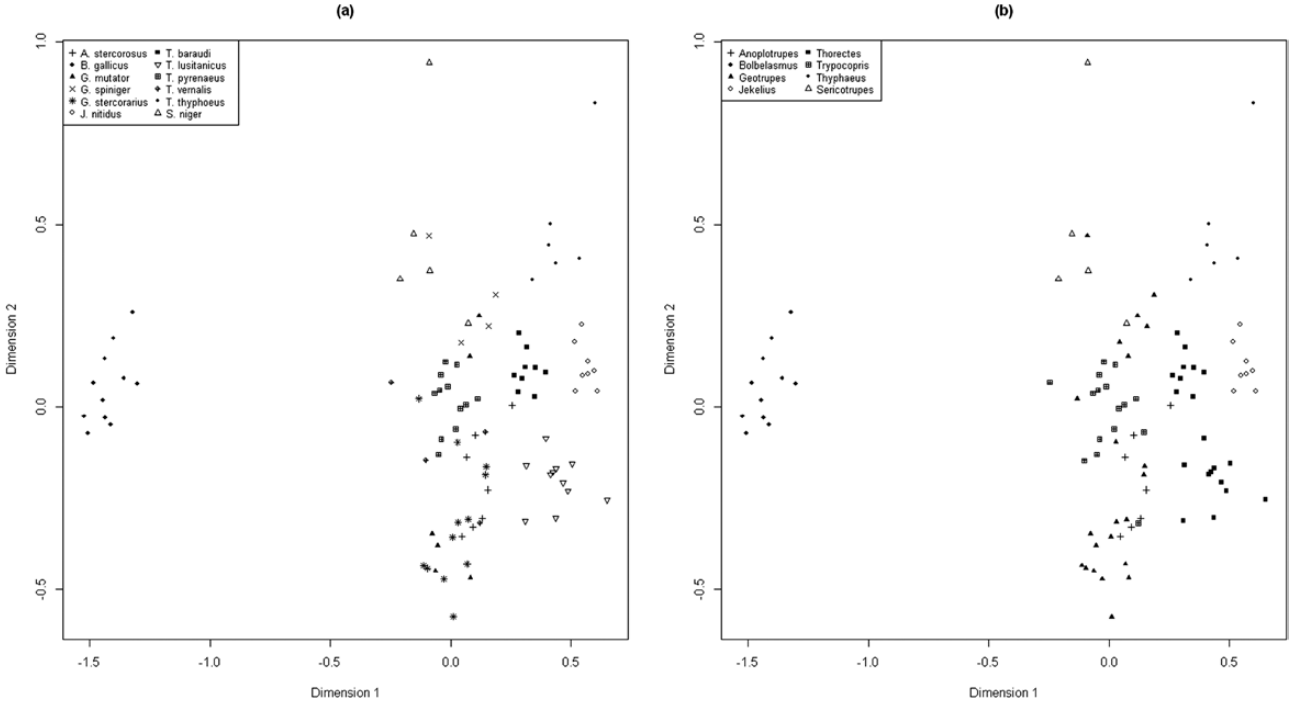

Fig. 2. Non-metric multidimensional scaling (stress value = 0.14) of the 69 cuticular compounds in the 12 studied species (a) and eight studied genius (b) of dung beetles using R 2.10.1 software.

Geotrupes (*G. stercorarius* and *G. spiniger*), were recovered as monophyletic. Our results suggest that *B. gallicus* is the most divergent, being highly separated from the other species (BV 100%). Furthermore, the Bolboceratidae family, represented in this study only by *B. gallicus*, occupied a basal position not only with respect to the remaining ingroup species (family Geotrupidae), but also in relation to the outgroup species (family Aphodiidae).

Discussion

The present study shows that the 12 studied species of dung beetle have distinct chemical profiles of CCs, and their use as systematic characters has demonstrated to be very useful to distinguish genera and species in this specific group of Coleoptera. These CCs are similar to those observed in other groups of insects (Gongyin et al. 2007, Fletcher et al. 2008, Baracchi et al. 2009). Our results demonstrated that the cuticular hydrocarbon profile of 7 of the 12 dung beetle species included in this study (namely, *B. gallicus*, *G. spiniger*, *J. nitidus*, *Ty. typhoeus*, *Tr. pyrenaeus*, *Th. baraudi*, and *Th. lusitanicus*) had a high taxonomic and diagnostic power. Moreover, only 10 compounds are sufficient to affiliate individuals to the correct species for at least six studied species. Our results also demonstrate that the chemotaxonomy is consistent with the mitochondrial and nuclear phylogenies for the majority of studied species.

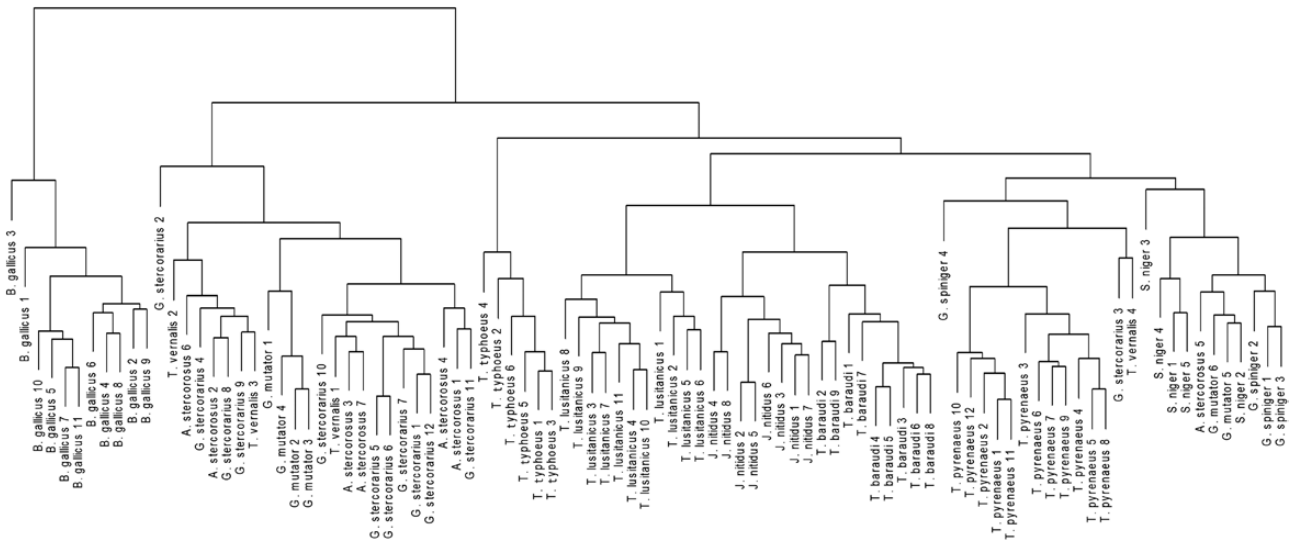

Fig. 3. Dendrogram for the 12 studied species of dung beetle species resulting from cluster analysis performed by average linkage between groups and using Euclidean distance and R 2.10.1 software. The relative distances among the individuals were determined by the distances among their chemical profiles.

Some authors consider the Bolboceratidae family to be a subfamily within the Geotrupidae (Lawrence and Newton 1995, Smith 2006). However, our chemical and molecular results support the separation of Geotrupidae and Bolboceratidae as distinct families (the latter family being represented by only one species in our study), which is in agreement with morphological studies (Browne and Scholtz 1999, Verdú et al. 2004). In addition, our molecular results support that the Bolboceratidae family, represented by *B. gallicus*, is the most divergent with respect to the Aphodiidae family. The sequence divergence and the phylogenetic distance in tree reconstructions showed that *B. gallicus* diverged from a common ancestral population at the same time than the Aphodiidae family and a long time before the Geotrupidae family. Our study demonstrates that chemical characters corroborate the genetic divergence for *B. gallicus*. Therefore, chemotaxonomy can be expected to be used as an important tool for evolutionary studies too. An unsophisticated extraction by solvent of compounds from an insect cuticle will straightforwardly allow to get species affinities when compare with the more time-consuming and expensive DNA extraction and analysis.

The chemotaxonomic model presented in this study was unable to discriminate between some species when the whole chemical profile was considered. For instance, our chemotaxonomic model was unable to distinguish between individuals of *G. stercorarius* from *G. mutator*, probably due to their close phylogenetic relationship. This observation underlined the fact that the use of chemical profiles for taxonomic and phylogenetic purpose is rapid, efficient, and cost-effective, but should be used as a consolidate with molecular tools as well. However, some single compounds by themselves can serve as a chemotaxonomic tool to distinguish between close species. For instance, the ratio of two compounds (CC39 and CC40) is higher, whereas the ratio of three other compounds (CC16, CC26, and CC33) is lower in *G. mutator* compared with *G. stercorarius*, allowing separation between both species. From a chemical point of view, *G. mutator* individuals appeared to be closer to *G. stercorarius* individuals than to *G. spiniger*, but *G. spiniger* and *G. stercorarius* individuals are closer and present in the same clade in the molecular phylogeny. This molecular result is in contradiction with the phylogeny by Cunha et al. (2011), where all *Geotrupes* species were monophyletic. However, using only the mitochondrial marker, we obtained a strong monophyly for all *Geotrupes* species (data not shown). We used the 18S nuclear gene, which seems to be less affected by *Geotrupes* speciation than the neurofibromin gene, used by Cunha et al. (2011).

Our chemotaxonomy and molecular phylogenies are congruent with the morphological taxonomy by Verdú et al. (2004) based on larval morphology. More than 30 morphological characters are needed to establish a phylogeny based on larvae. Thereby, to simplify the approach, it could be interesting to study the dung beetle chemotaxonomy using larval cuticular profiles.

The CCs are known to have a functional role as kairomones to trigger phoretic interactions: they are used by phoretic mites to discriminate their host-carrier among several candidates, even within the same genus (Niogret et al. 2006, 2010). However, mating behavior in dung beetles is not well documented, most probably because males and females are primarily attracted by food resources and do not require other chemical cues to find a mate (Price and May 2009). However, in the field, some dung pats attract many individuals of the same species as, e.g., the case of *G. spiniger* or *G. stercorarius*, whereas nearby similar pats contain no beetles (J.-P. Lumaret, personal observation). This aggregation effect may be due to chemical cues other than a food resource, as shown by Ortiz-Domínguez et al. (2006). Our study has shown that each of the studied species can be differentiated by their cuticular profile.

Conclusion

Our study demonstrated that CC profiles are powerful diagnostic tools for species separation among dung beetles. Moreover, CC extraction and analysis could be a simplified and less expensive way to distinguish species when compared with current molecular techniques. Chemical ecology and molecular biology are two main scientific approaches to study insect interactions. The combination of those different techniques can improve our ability to clarify complex hypotheses and evolutionary scenarios that single techniques fail to resolve.

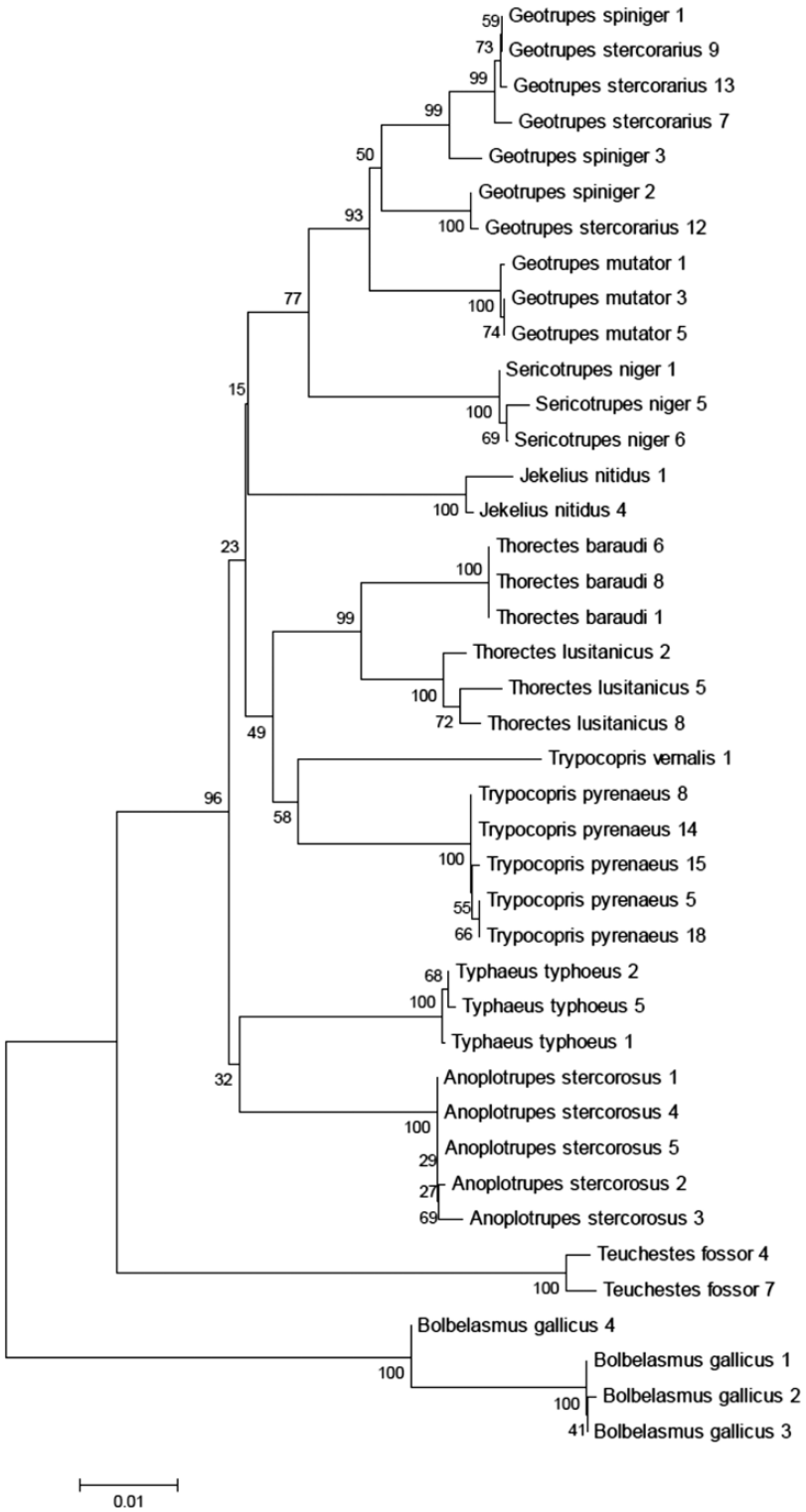

Fig. 4. Neighbor-joining analysis phylogenetic tree for the concatenation of mitochondrial and nuclear used markers. Numbers at nodes indicate the statistical support as obtained from 1,000 bootstrap replicates.

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