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Cuticular Hydrocarbon Profiling Reveals Chemotaxonomic Diversity among *Gonipterini weevils* (Coleoptera: Curculionidae) †

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Abstract: Cuticular hydrocarbons (CHCs) have been used as a chemotaxonomic tool to support the classification and identification of various insect species for decades. However, there have been limited research performed on the CHC profiles of weevils (Coleoptera: Curculionidae), despite the extensive diversity and ecological significance of this family. In this study, CHCs were extracted from fifteen Gonipterini weevil specimens from eastern Australia, comprising five species from three genera. Analysis by gas chromatography-mass spectrometry (GC-MS) revealed the presence of over 90 compounds, with the most abundant compounds including nonacosane, 7-methylheptacosane, heptacosane and hexacosane. Principal component analysis revealed *Bryachus squamicollis* to be the most dissimilar species in terms of its CHC profile, while the two *Oxyops* species showed relatively similar CHC profiles. The results may support the use of CHC profiling as a chemotaxonomic tool for the identification and delineation of various Gonipterini genera and species.

Keywords: *Gonipterus*; *Oxyops*; *Eucalyptus*; chemotaxonomy

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1. Introduction

The Gonipterini tribe of weevils (Coleoptera: Curculionidae) contains a number of economically important species, including several species of *Gonipterus* which have become international pests of *Eucalyptus* plantations [1,2] and *Oxyops vitiosa*, which has been used for the biocontrol of the invasive species *Melaleuca quinquenervia* in the Florida everglades [3]. Consequently, the accurate identification of species from this tribe is of utmost importance. However, *Gonipterus* in particular contains a number of cryptic species, the identification of which typically requires molecular analysis and/or dissection of male genitalia [2].

However, the emergence of cuticular hydrocarbon (CHC) profiling has emerged as a useful chemotaxonomic tool over the past few decades [4]. These compounds are endogenously synthesized by nearly all insects and exported to the cuticle [5,6], with one of their major functions being to mediate communication with other insects. Consequently, there is a wide range of diversity in CHC profiles, which is reflective of the genetic diversity of the species in question. Although CHC profiling has been used to support the classification and discrimination of other beetle species [7,8], there have been limited studies to date on the weevil family, despite the large number of species present.

Recently, Souza, et al. [9] demonstrated that the differences in CHC profiles between a number of *Gonipterus* species concurred with molecular sequencing data and morphological features, demonstrating that this method may be used for the rapid classification of species from this genus. However, these authors did not examine the CHC profiles of other Gonipterini genera in detail, only using *Oxyops* (one or more unidentified species) as an outgroup. Specifically, there has been no work to date looking at the CHC profiles of different *Oxyops* or *Bryachus* species. In order to fill this research gap, the present study aimed to extend the use of CHC profiling to different genera and species from the Gonipterini tribe.

2. Materials and Methods

In February 2021, 15 weevils, comprising three genera and five species, were collected from *Eucalyptus populnea* saplings on a Central Queensland grazing property (23°46′ S, 150°21′ E). Details of the specimens are provided in Table 1, while scale photographs are given in Figure 1. CHCs were extracted from each weevil using 300 µL of hexane with 4 min of agitation, following the methods of Souza, et al. [9]. The hexane extracts were analysed by gas chromatography-mass spectrometry (GC-MS), following the methods of Souza, et al. [9], but using a single quadrupole Shimadzu QP2010 Plus system fitted with a Shimadzu SH-Rxi-5Sil MS column (29 m × 0.25 mm i.d. × 0.25 µm thickness). Compounds were identified from comparison of their mass spectra and linear retention indices (LRIs; calculated from a series of C_8–C_{40} alkanes run under the same conditions) to literature values [10–12]. Data analysis was conducted in R studio [13].

Table 1. Details of the Gonipterini weevil specimens analysed in this study.

Species	No. Specimens	Length (mm)	Width (mm)
Oxyops fasciculatus	5	6.4 ± 0.4	3.4 ± 0.2
Oxyops sp. (undescribed)	3	7.6 ± 0.5	4.1 ± 0.3
Gonipterus sp. n. 2 (tentative ID)	1	7.8	4.2
Gonipterus cinnamomeus	3	6.6 ± 0.2	3.7 ± 0.2
Bryachus squamicollis	3	9.9 ± 0.8	5.0 ± 0.5

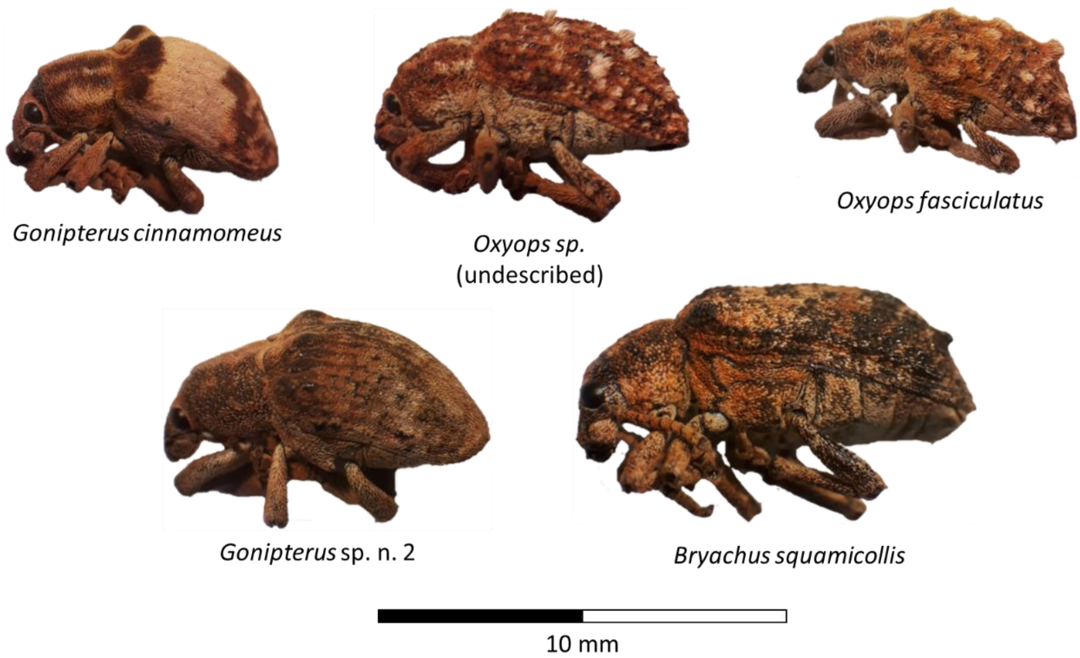

Figure 1. The five Gonipterini weevil species investigated in this study. Photographs are to scale.

3. Results and Discussion

3.1. CHC Profiles

A total of 97 compounds were found across the five weevil species, with 59 of these identified from their mass spectra and LRI, and a further 24 compounds tentatively identified from their mass spectra where their LRIs could not be found in the literature. Table 2 lists the identified compounds, along with their relative abundance in each of the weevil species. This number of compounds was significantly more than the 31 CHCs identified by Souza, et al. [9], likely due to the wider range of genetic diversity sampled here (i.e., three different Gonipterini genera instead of two). In addition, compounds eluting earlier than icosane (C_{20}) were considered in this study.

The most abundant compounds across all five species were nonacosane and 7-methylheptacosane. Notably, Souza, et al. [9] had not reported these CHCs from their work on *Gonipterus* species. As both of these compounds were identified from their mass spectra and LRIs, a high level of confidence can be had in the identities reported here.

B. squamicollis also contained high levels of heptacosane, while *Oxyops* sp. contained higher concentrations of 2-methyloctacosane, and both *Gonipterus* species contained high levels of hexacosane, comparative to the levels reported by Souza, et al. [9] for several *Gonipterus* species. *Oxyops* sp. also contained much lower concentrations of 7-methylheptacosane compared to the other species.

Notably, very low levels of 2-methylhexacosane were found in the species studied here; whereas Souza, et al. [9] found relatively high concentrations of this compound in their species of *Gonipterus* (although it was not detected in *Oxyops*).

3.2. Chemometric Analysis

In order to visualize the broad differences in CHC composition between species, principal component analysis (PCA) was performed on the normalized CHC dataset. As can be seen from the scores plot in Figure 2, *B. squamicollis* was well separated from the remaining species, while the *Gonipterus* specimens were located toward the centre of the plot. The undescribed *Oxyops* species showed some overlap with *O. fasciculatus*, although it was slightly more separated across the third principal component (data not shown).

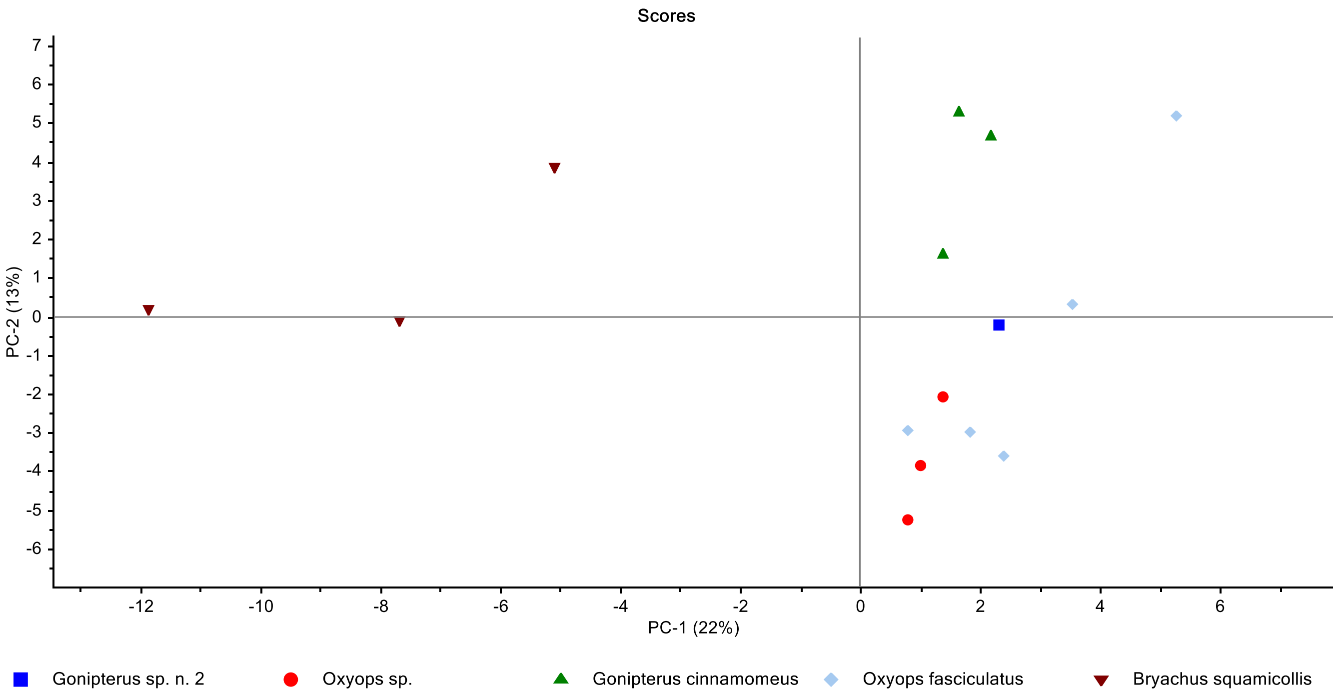

Figure 2. Scores plot showing the results of PCA performed on the normalized CHC data.

Table 2. Compounds identified from the Gonipterini specimens using GC-MS. The abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample.

No.	Compound	LRI	M⁺ (*m/z*)	Ident. ^	*B. squamicollis* (n = 3)	*G. cinnamomeus* (n = 3)	*G.* sp. n. 2 (n = 1)	*O. fasciculatus* (n = 5)	*Oxyops* sp. (n = 3)
1	3-hexanone	787	100	MS, LRI	0.03 ± 0.01	0.06 ± 0.01	0.08	0.05 ± 0.04	0.04 ± 0.00
2	2-hexanone	791	100	MS, LRI	0.02 ± 0.02	0.07 ± 0.01	0.07	0.06 ± 0.04	0.04 ± 0.01
3	2,4-dimethylheptane	820	128	MS, LRI	0.02 ± 0.01	0.02 ± 0.02	0.05	0.03 ± 0.02	0.02 ± 0.00
4	Heptanal	902	114	MS, LRI	0.03 ± 0.01	0.03 ± 0.02	0	0	0
5	Octanal	1002	128	MS, LRI	0.01 ± 0.01	0	0	0	0
6	Eucalyptol	1033	154	MS, LRI	0	0	0.10	0.01 ± 0.02	0
7	3,6-dimethyldecane	1055	170	MS, LRI	0.03 ± 0.01	0.06 ± 0.02	0.06	0.05 ± 0.03	0.03 ± 0.01
8	2,6,8-trimethyldecane	1099	184	MS, LRI	0.02 ± 0.02	0.02 ± 0.03	0	0.01 ± 0.03	0.01 ± 0.01
9	Nonanal	1104	142	MS, LRI	0.13 ± 0.05	0	0	0	0
10	Decanal	1205	156	MS, LRI	0.01 ± 0.01	0.02 ± 0.04	0	0	0
11	Exo-2-hydroxycineole	1228	170	MS, LRI	0.04 ± 0.02	0.08 ± 0.04	0	0.02 ± 0.02	0
12	Tentative: 2,6,10-trimethylundecane	1275	198	MS, LRI	0.03 ± 0.01	0.06 ± 0.01	0.05	0.06 ± 0.03	0.03 ± 0
13	10-undecenal	1282	168	MS, LRI	0.14 ± 0.17	0	0	0	0
14	Tentative: carvacrol	1297	150	MS, LRI	2.42 ± 3.83	0.04 ± 0.07	0	0.02 ± 0.04	0
15	Tentative: isoascaridole	1312	168	MS, LRI	0.01 ± 0.01	0	0	0	0
16	Tentative: 4a-methyldecahydro-1-naphthalenol	1319	168	MS, LRI	0.21 ± 0.23	0	0	0	0
17	4,6-dimethyldodecane	1321	198	MS, LRI	0.03 ± 0.01	0.02 ± 0.03	0	0.03 ± 0.03	0.01 ± 0.02
18	Tentative: *cis-p*-menth-1-en-3,8-diol	1358	170	MS, LRI	0.02 ± 0.03	0	0	0	0
19	(+)-*cis,trans*-nepetalactone	1364	166	MS, LRI	0.02 ± 0.03	0	0	0	0
20	Dodecanal	1408	184	MS, LRI	0	0.02 ± 0.03	0	0.01 ± 0.01	0.01 ± 0.02
21	Aromadendrene	1444	204	MS, LRI	0	0	0	0.01 ± 0.03	0
22	Unidentified alkane 1	1488	-	MS	0.01 ± 0.01	0.01 ± 0.02	0.06	0.05 ± 0.02	0.03 ± 0.00
23	Bicyclogermacrene	1501	204	MS, LRI	0.03 ± 0.05	0	0	0.02 ± 0.02	0
24	Tentative: 2,6,10-trimethyltridecane	1534	226	MS, LRI	0.01 ± 0.01	0.01 ± 0.02	0	0	0
25	Globulol	1592	222	MS, LRI	0	0	0.07	0.01 ± 0.03	0
26	Tetradecanal	1612	212	MS, LRI	0.04 ± 0.02	0	0.05	0.01 ± 0.02	0.02 ± 0.03
27	Heptadecane	1699	240	MS, LRI	0.03 ± 0.01	0.05 ± 0.01	0.05	0.04 ± 0.03	0.02 ± 0.02
28	Phytane	1743	282	MS, LRI	0.01 ± 0.02	0	0	0	0
29	*Cis*-9-hexadecenal	1795	220	MS, LRI	0.05 ± 0.01	0	0	0	0
30	Hexadecanal	1816	240	MS, LRI	0.17 ± 0.05	0.09 ± 0.04	0.10	0.16 ± 0.13	0.13 ± 0.08
31	6,10,14-trimethyl-2-pentadecanone	1840	268	MS, LRI	0	0	0	0	0.01 ± 0.02
32	2-heptadecanone	1899	254	MS, LRI	0.09 ± 0.02	0.07 ± 0.03	0.14	0.04 ± 0.05	0.06 ± 0.02
33	Tentative: 2,2-dimethyloctadecane	1910	282	MS, LRI	0.04 ± 0.02	0.06 ± 0.06	0.09	0.03 ± 0.03	0.02 ± 0.03
34	Heptadecanal	1918	254	MS, LRI	0.05 ± 0.01	0	0	0	0

35	Tentative: 3-ethyl-3-methylheptadecane	1953	283	MS, LRI	0.03 ± 0.02	0.06 ± 0.02	0.05	0.06 ± 0.03	0.03 ± 0.00
36	9-octadecanone	1990	268	MS, LRI	0	0	0	0.1 ± 0.1	0
37	*Cis*-13-octadecenal	1995	266	MS, LRI	0.12 ± 0.03	0	0	0	0
38	Tentative: *cis*-9-octadecenal	2014	266	MS, LRI	0	0	0	0.03 ± 0.07	0
39	Octadecanal	2019	268	MS, LRI	0.61 ± 0.14	0.07 ± 0.04	0.13	0.19 ± 0.18	0.16 ± 0.13
40	*Cis*-2-octadecen-1-ol acetate	2074	310	MS, LRI	0	0	0	0.01 ± 0.01	0
41	2-nonadecanone	2098	282	MS, LRI	0.09 ± 0.02	0.04 ± 0.04	0.18	0.02 ± 0.05	0.08 ± 0.05
42	Nonadecanal	2117	282	MS, LRI	0.06 ± 0.01	0	0	0	0
43	Unidentified alkane 2	2128	-	MS	0.23 ± 0.21	0.25 ± 0.43	0	0.03 ± 0.07	0.12 ± 0.22
44	Unidentified alkane 3	2139	-	MS	0	0.35 ± 0.60	0	0	0.23 ± 0.40
45	Unidentified alkane 4	2148	-	MS	0.48 ± 0.46	0.42 ± 0.73	0	0.06 ± 0.14	0.28 ± 0.48
46	Unidentified alkane 5	2160	-	MS	0.10 ± 0.08	0.14 ± 0.24	0.07	0.04 ± 0.02	0.01 ± 0.02
47	Unidentified alkane 6	2168	-	MS	0.08 ± 0.08	0.09 ± 0.15	0	0	0.28 ± 0.49
48	Docosane	2197	310	MS, LRI	0.02 ± 0.02	0	0	0.06 ± 0.05	0
49	Eicosanal	2222	296	MS, LRI	0.09 ± 0.04	0	0.71	0.03 ± 0.07	0
50	Unidentified alkane 7 [a]	2260	-	MS	0	0	0	0	0.03 ± 0.03
51	Tricosane	2297	324	MS, LRI	0.74 ± 0.19	0.37 ± 0.09	0	1.35 ± 0.54	0.27 ± 0.06
52	Unidentified ketone 1	2304	-	MS	0	0	0.09	0	0
53	Henicosanal	2326	310	MS, LRI	0	0	0.50	0	0
54	11-methyltricosane	2331	338	MS, LRI	0.20 ± 0.34	0	0	0	0.02 ± 0.04
55	Unidentified aldehyde	2367	-	MS	0.02 ± 0.01	0	0	0	0
56	3-methyltricosane	2374	339	MS, LRI	0.02 ± 0.03	0	0	0	0
57	Tetracosane	2400	338	MS, LRI	0.03 ± 0.02	0.07 ± 0.01	0.16	0.09 ± 0.05	0.02 ± 0.02
58	Docosanal	2430	324	MS, LRI	0.01 ± 0.01	0.58 ± 0.31	1.12	0	0
59	Tentative: 9-methyltetracosane	2437	352	MS, LRI	0.01 ± 0.02	0	0	0	0
60	2-methyltetracosane	2473	352	MS, LRI	0	0.94 ± 1.64	0	0	0.69 ± 1.19
61	Tentative: x-pentacosene	2479	352	MS, LRI	0	0	0	0.03 ± 0.07	0
62	Pentacosane	2499	352	MS, LRI	1.67 ± 0.93	1.83 ± 0.37	1.82	4.26 ± 2.02	0.97 ± 0.88
63	Unidentified ketone 2 [b]	2509	-	MS	0	0.05 ± 0.09	0.13	0	0
64	Tentative: 7-methylpentacosane	2522	367	MS, LRI	0	0.27 ± 0.46	0	0.48 ± 0.42	0
65	Tentative: 11-methylpentacosane	2530	395	MS, LRI	0	0.15 ± 0.26	0	0.11 ± 0.14	0
66	Tentative: 13-methylpentacosane	2569	367	MS, LRI	0.03 ± 0.05	0	0	0	0
67	3-methylpentacosane	2574	366	MS, LRI	0.26 ± 0.23	1.09 ± 0.64	0.27	0	0
68	Tentative: 11,15-dimethylpentacosane	2584	409	MS, LRI	0	0	0	0.70 ± 1.57	0
69	Hexacosane	2600	366	MS, LRI	9.04 ± 6.87	13.26 ± 7.6	14.70	8.30 ± 7.10	11.01 ± 8.76
70	Tetracosanal	2637	352	MS, LRI	1.95 ± 2.56	4.22 ± 0.91	0	0	0.16 ± 0.27
71	2-methylhexacosane	2663	380	MS, LRI	0	0	0	0.03 ± 0.07	0
72	Unidentified alkane 8	2672	-	MS	0	0.51 ± 0.46	0	0	0.15 ± 0.15

73	Tentative: 13-methylhexacosane	2682	381	MS, LRI	0.65 ± 1.12	0.02 ± 0.04	0	0.88 ± 1.37	0
74	Unidentified alkane 9	2690	-	MS	0.36 ± 0.62	0	0	0	0
75	Heptacosane	2704	380	MS, LRI	11.32 ± 2.95	4.3 ± 4.94	2.27	1.54 ± 3.44	0.36 ± 0.62
76	Tentative: 7-methylheptacosane	2712	395	MS, LRI	21.49 ± 5.33	28.94 ± 7.24	25.45	27.58 ± 6.29	16.77 ± 6.68
77	Unidentified ketone 3	2723	-	MS	0	0.75 ± 0.31	0	0	0
78	13-methylheptacosane	2737	394	MS, LRI	0.32 ± 0.06	0.07 ± 0.13	0	0.08 ± 0.15	0.73 ± 0.24
79	Unidentified alkane 10	2755	-	MS	1.14 ± 1.2	0	0	0.01 ± 0.02	0.10 ± 0.17
80	Tentative: 11-methylheptacosane	2759	395	MS, LRI	0	4.60 ± 4.16	0	1.18 ± 2.49	0.14 ± 0.23
81	2-methylheptacosane	2764	394	MS, LRI	0	0	0	0	0.08 ± 0.07
82	Docosyl pentyl ether	2770	396	MS, LRI	0	0	0	0	0.27 ± 0.47
83	3-methylheptacosane	2774	394	MS, LRI	0	3.14 ± 1.61	2.82	0	0
84	Tentative: 5,15- or 5,17-dimethylheptacosane	2777	409	MS, LRI	1.06 ± 1.07	0	0	0	0
85	Tentative: 5,11-dimethylheptacosane	2784	409	MS, LRI	0.36 ± 0.62	1.93 ± 3.35	0	3.93 ± 7.21	0.79 ± 1.37
86	Octacosane	2800	394	MS, LRI	6.89 ± 2.27	3.55 ± 0.89	19.10	4.16 ± 0.85	4.43 ± 1.34
87	Squalene	2811	384	MS, LRI	0.38 ± 0.65	0.36 ± 0.37	0.71	0.43 ± 0.20	0.27 ± 0.08
88	Tentative: 12-methyloctacosane	2830	409	MS, LRI	0.22 ± 0.14	0.05 ± 0.08	0	0	0
89	Hexacosanal	2837	380	MS, LRI	0.94 ± 0.24	0.94 ± 0.3	0	0	0
90	Tentative: x-methyloctacosane	2858	408	MS, LRI	0.05 ± 0.05	0	0.11	0	0
91	2-methyloctacosane	2865	408	MS, LRI	0.24 ± 0.14	0.93 ± 0.22	0.96	0	8.28 ± 6.05
92	Nonacosene	2881	407	MS, LRI	1.03 ± 0.40	0	0	0	0
93	1-hexacosanol	2890	382	MS, LRI	0	0.21 ± 0.20	0	0	0
94	Nonacosane	2918	408	MS, LRI	26.54 ± 5.05	17.34 ± 3.08	24.90	42.99 ± 14.09	51.41 ± 13.58
95	Triacontane	2982	422	MS, LRI	5.72 ± 0.63	7.31 ± 3.07	2.79	0.51 ± 0.37	1.38 ± 0.68
96	Tentative: x,12-dimethylnonacosane	3002	437	MS, LRI	0.30 ± 0.50	0	0	0.01 ± 0.02	0
97	Tentative: 2-methyltriacontane	3039	437	MS, LRI	1.38 ± 0.43	0	0	0	0

^ Identification methods: LRI = linear retention index; MS = mass spectra. [a] “Undetermined B” from Souza, et al. [9]. [b] May be “Undetermined G” from Souza, et al. [9].

Hierarchical cluster analysis, performed on the normalized GC-MS data using the Euclidean distance with Ward's method of clustering, displayed similar results. *Bryachus squamicollis* was again identified as the outlier taxon, while the two *Oxyops* species were most closely related in terms of their CHC composition (Figure 3).

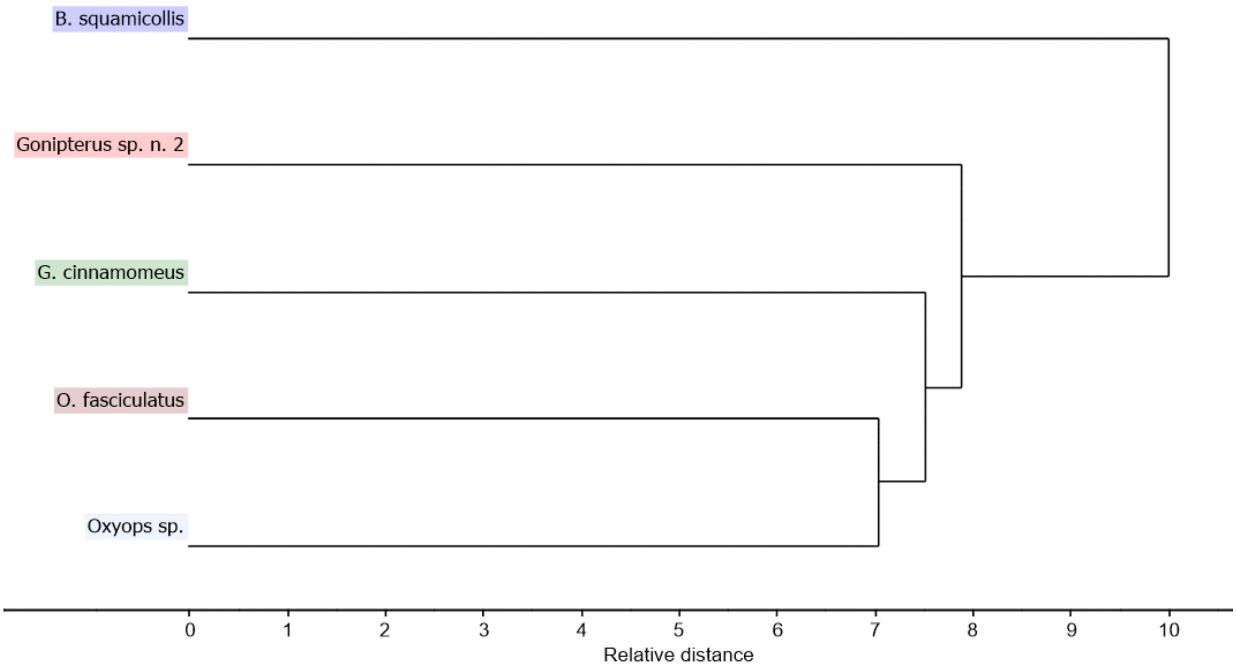

Figure 3. Hierarchical cluster analysis performed on the normalized CHC data.

4. Conclusions

The results of this proof-of-concept study support the use of CHC profiling as a potential chemotaxonomic method for discriminating between different genera and species of Gonipterini weevil. Around three times as many compounds were identified and quantified compared to previous work focusing on *Gonipterus* [9], with some of identities of the major CHCs differing from those found in previous work on the Gonipterini. As suggested by Souza, et al. [9], it may be possible to extract CHCs from live specimens in the future, further increasing the usefulness of this technique. However, further investigation with a larger number of specimens is recommended.

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